

In vitro Pearl Culture Techniques

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FOREWORD

India is one of the renowned pearl producing countries in the world. Gulf of Mannar which lies between the south eastern tip of India and the west coast of Sri Lanka, is known for its rich biodiversity and pearl banks. The natural pearls produced by the pearl oysters at the Tuticorin side were more lucrative and considered to be the best pearls compared to the pearls from other countries. In recognition of its pearl fishery, Tuticorin city in Tamil Nadu is named as 'Pearl City'. Due to over exploitation of pearl oysters, the government had banned pearl fishery. The last pearl fishery in the Gulf of Mannar was conducted in 1961. The main reason for the failure of pearl fishery was due to non-revival of pearl oyster population in the natural beds which may be due to environmental impact. Therefore the availability of natural pearls has become a rarity.

The efforts taken up by the Central Marine Fisheries Research Institute (CMFRI) to produce cultured pearls through in-vivo technique, utilizing the available pearl oyster resources of the beds succeeded in producing pearls in 1973 for the first time in the country.

Tissue culture project for the production of laboratory (in-vitro) pearls under controlled conditions was initiated by CMFRI to face the challenges of growing environmental pollution. Japan is the pioneer country in developing tissue culture technology using marine organisms in 1970's itself. India too was interested in tissue culture technology and so Dr. S. Dharmaraj, a scientist from pearl culture team was sponsored to Japan to undergo an advanced training in marine invertebrate tissue culture in 1985. The Japanese government agreed to provide training on the culture of mantle tissue of a bivalve other than pearl oyster.

The basic in-vitro technology learnt from Japan helped us to establish the first ever tissue culture laboratory for marine organisms in India. Dr. S. Dharmaraj and Dr. C.P. Suja initiated the work at the Tuticorin Research Centre of CMFRI in 1996.

In-vivo cultured pearl production provides no certainty of pearl quality. The in-vitro technology affords ample chances for manipulation of pearl quality, unlike in-vivo production where the process is totally governed by extrinsic and intrinsic factors.

The in-vitro technology now developed at CMFRI can be extended to other pearl producing molluscs. It has the potential to pave the way for the production of pearls of various colours from different species of molluscs.

I am hopeful that this book will serve as the basis to understand the complexity of nacre for students and researchers. With further refinement and up gradation, the day is not far off when high-tech tissue culture laboratories would be able to produce marine pearls of desired shape, size and colour.

The progress made so far by CMFRI is only a small beginning and the institute looks forward to reach greater heights in the days to come.

Dr. A. Gopalakrishnan
Director, CMFRI

January 2019

PREFACE



The involvement of cell and tissue culture to the advancement of agriculture, biological and medical field is universally known. The use of tissue culture is expanded in many ways with biological experimentations dealing with humans and vertebrates. But the invertebrate tissue culture is limited to some insect and a few molluscan species.

This book deals with the specific and exclusive area of tissue culture of mantle cells of pearl producing molluscs with the aim to produce *in-vitro* pearl. At present, pearl production is undertaken by farming in the sea by *in-vivo* methods. It involves huge sum of money in terms of farm materials, farm management, labour cost, fuel cost, machinery cost etc. In spite of all, no manipulation is possible for pearl quality. The *in-vitro* technology waives all such expenditures and open sea farming could be totally avoided. Once the technology is commercialized, a possibility of pearl revolution would take place at global level.

This book deals with a detailed study of different aspects of mantle cells such as dissociation, type, migration, behaviour and multiplication. This study brought a clear clue with regard to type of cells, their functions in pearl sac formation and crystal deposition which formed the basis for *in-vitro* pearl formation. Surprisingly, the results are identical to the studies on *in-vivo* shell regeneration. In this specific study, the techniques and methodology used for pearl sac formation are unique.

Apart from producing pearls, there are wide applications using the cell culture systems for cytogenetic, histochemical and biomedical applications. As nacre is considered suitable biomaterial due to its mechanical properties, this *in-vitro* study of mantle cells responsible for nacre, will serve as a guide for tissue engineers for forming a new biocompatible biomaterial.

At this juncture, I owe my deep debt of gratitude to late Dr. M. Devaraj, former Director of CMFRI who gave me the opportunity to work in this challenging project. I would like to thank the former directors, Dr. Mohan Joseph Modayil and Dr. G. Syda Rao for their continued support and encouragement. I thank, Heads of Divisions and Scientists-in-charge of Tuticorin Research Centre for their help during this study. The freedom for work and guidance given by Dr. S. Dharmaraj is the basis for the entire work. I record my gratitude for the support and encouragement given

by Dr. A. Goplalakrishnan, Director, CMFRI. I am grateful to Dr. P. Vijayagopal, Head of Division for his guidance for editing the book, Dr. P. P. Manojkumar, Scientist in charge of Tuticorin centre for his support and Dr. I. Jagadis, Principal Scientist, Molluscan Division for the identification of molluscan species. I am indebted to late Dr. P. Muthiah, former Scientist in charge of the Centre for his timely help. It is my pleasure to thank Dr. K. K. Vijayan, former HOD, MBTD, CMFRI and Director, CIBA for strengthening our team by including Dr. Vidya Jayasankar and Dr. Srinivasa Raghavan. I am extremely happy that I have trained them to take up this work to greater heights. I would like to thank all authors for their contributions and Dr. S. Lakshmana Senthil for his continuous assistance throughout the preparation of this book. I am thankful to Ms. Koncies Mary, Technician and all staff for their help during this study. I wish to thank my husband, Mr. P. George Thomas, my family members and friends for their constant encouragement and support.

Dr. C.P. Suja

Principal Scientist

January 2019

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Chapter 1

ORIGIN OF TISSUE CULTURE FOR PEARL PRODUCTION AND THE BASIC SET UP FOR THE LABORATORY

S. Dharmaraj and C.P. Suja

Background

India is one of the famous pearl producing countries in the world. It is bestowed with pearl oyster population in the natural pearl banks of Gulf of Mannar in South-West coast of Tuticorin. The natural pearls produced by the pearl oysters from the Gulf of Mannar and Persian Gulf are considered to be the best 'Oriental Pearls' and are highly valuable than pearls from other countries. Tuticorin in Tamil Nadu was once a converging point for natural pearls taken from pearl oysters from the sea bed by organizing periodic pearl fishery. Due to the abundance of famous 'Oriental Pearls', Tuticorin city is named as 'Pearl City'. The last pearl fishery was conducted in 1961 in the Gulf of Mannar and in 1966 in Gulf of Kutch. The main reason for the failure of pearl fishery is due to non-revival of pearl oyster population in the natural beds due to environmental impact. Therefore the availability of natural pearls has become a rarity.

There are many myths and theories on the origin and formation of pearl. Tokichi Nishikawa in 1907 gave the most reasonable scientific explanation for this (Cahn, 1949; Alagarwamy, 1970). His theory known as the pearl-sac theory which explains that the pearl secreting cells of the mantle (the outer most soft covering of the molluscs), migrate into the body of the oyster under the stimulus of a foreign body and form a pearl sac by cell division around the foreign body; the pearl sac secretes nacre which gets deposited on the foreign body and in the course of time a pearl is produced (Alagarwamy, 1987). The pearl produced by the pearl oyster by natural process is called 'natural pearl' and it is accidental and very rare. Pearls produced by human interference by introducing shell beads as foreign body through surgical operation are cultured pearls. In order to revive the glory of Indian pearls, the Central Marine Fisheries Research Institute (CMFRI) initiated efforts to produce cultured pearls, utilizing the available pearl oyster resources of the beds in 1972 and succeeded in producing cultured pearl in 1973 for the first time in the country (Alagarwami, 1974). Commercialization of pearl culture was not possible due to non availability of wild stock and high cost in the collection of the same. In view of non-productive wild stock, hatchery technology was developed to augment sustained supply of oysters for pearl culture. The survival of pearl oyster stock faces uncertainty in view of viral diseases as it had happened in Japan, other fishing gears destroying the ecosystem, increasing environmental pollution due to industries and expansion of harbours.

This has caused great concern to pearl culture entrepreneurs throughout the world. Culturing of pearls in natural environment with ever increasing levels of pollutants is not feasible. CMFRI, the pioneers in pearl production technology in India, initiated mantle tissue culture of pearl oyster with an aim of *in vitro* pearl production by explant and organ culture technology.

Explant culture of mantle tissue is a tissue culture technique to culture cells from a piece or pieces of mantle tissue which is responsible for nacre secretion. The organ culture of mantle tissue is the culture and maintenance of a part of the tissue or organ removed from the oyster to allow differentiation and preservation of the architecture and function. Both the techniques are used to study the *in-vitro* pearl formation.

Laboratory set-up for tissue culture

The culture room is the vital portion of the tissue culture laboratory where the preparation, processing of tissues, inoculation and incubation of explant and maintenance of cultures are carried out. Very strict hygienic condition is essential. All infrastructures are strictly maintained in sterile condition. The atmospheric air in the culture room is sterilized by UV irradiation and free entry into the room is restricted. Foot wear from other rooms is prohibited and a set of footwear is made available in the room. Utmost care is taken to clean the room with antiseptic solution and fumigation is carried out periodically. At the time of organisation of cultures, research workers wear sterile aprons, facemask with head cap and surgical gloves to avoid contamination. The room temperature is controlled by split air conditioners.

The laboratory space is divided into different modules (Fig.1.1) with specific functions and uses.

- ❖ Entrance Room
- ❖ Preparation Room
- ❖ Pass Box
- ❖ Dressing Room
- ❖ Clean Room

Entrance Room

Strict protocols are maintained for entering into the Lab Entrance Room.

- ❖ Those who are entering into the lab should leave their footwear outside.
- ❖ All should dip their feet in the chlorinated water placed in a trough in front of the room.

- ❖ They should dry their feet on the mat placed at the entrance room.
- ❖ People from outside are allowed to enter only up to this room.

Preparation Room

- ❖ Person entering into the lab should wear the footwear placed inside the room.
- ❖ This room is equipped with
 - q Balance
 - q pH meter
 - q Freezer
 - q Refrigerator
 - q Water Purifier
 - q Centrifuge
 - q Water Bath
 - q Autoclave
- ❖ This room is meant for cleaning, drying, storing, preparation and sterilization of glassware and instruments.

Pass box

All the sterilized items are stored in this box. It is provided with UV light and this light is switched on for 20 minutes before the sterilized items are taken into the clean room.

Dressing Room

It is the place for wearing sterile cloths before entering the clean room.

Clean room

- ❖ Strict aseptic conditions are maintained in this room.
- ❖ Clean room is equipped with
 - q Laminar flow
 - q CO₂ incubator
 - q Ultra centrifuge
 - q Inverted phase contrast microscopes
 - q UV Lights
- ❖ The UV lights are switched on for 20 minutes before the culture work. The inoculation, incubation and observations are done in this room.



Fig. 1.1 Tissue culture Laboratory:

A) Entrance Room B) Preparation Room C) Pass Box D) Dressing Room E) Clean Room

Sterilization Protocols

Room

- ❖ Room is mopped with disinfectant solution twice in a week and kept clean always.
- ❖ Switch on the UV light in the clean room for twenty minutes before culture starts.
- ❖ Wipe all surfaces of tables and instruments with 70% alcohol periodically.

Cleaning of glassware and instruments

- ❖ Cleaning and sterilization are most important aspects in tissue culture.
- ❖ Success of tissue culture depends on a very high degree of cleanliness and thorough sterilization of instruments and other materials used.
- ❖ Slightest defect in cleaning and sterilization will affect tissue culture drastically.

Cleaning Procedures

- ❖ Glass ware and other materials after use on clean bench are washed in fresh water to remove adhering substances and solutions.
- ❖ Soaked in soap water for 24 hours.
- ❖ Each material is cleaned in fresh water for at least eight times.
- ❖ Glass pipettes are washed in automatic pipette washing unit.
- ❖ Washed materials are rinsed twice in glass distilled water and once with double distilled water.
- ❖ Washed materials are shaken vigorously to remove water particles and then dried at room temperature.
- ❖ Clothes and face masks are washed in detergent powder and dried in sunlight.

Preparation, Sterilization and Storing of Materials

- ❖ Mouth of the beakers, measuring cylinders is closed with aluminium foil.
- ❖ Pipettes and droppers are stored by inserting a piece of cotton at head portion.
- ❖ Clothes and face mask are properly folded and stored.
- ❖ Instruments like forceps, scissors, knife, scalpel etc. are wrapped in aluminium foil individually and stored in a metal box.
- ❖ Petri dishes, glass bowls and droppers are covered with cellophane paper.
- ❖ All the packed items are sterilized by dry or wet methods.
- ❖ The sterilized items are stored in PassBox.

Collection of animals

The pearl oysters are collected from the rafts in the farm maintained by CMFRI in the harbour region. Other pearl producing molluscs used in the experiments are collected from Kayalpattanam landing centre of Gulf of Mannar. Abalones are collected from Tuticorin Harbour area underneath the granite stones. The abalones prefer inter tidal zone where the artificially laid stones have luxurious growth of green algae. Collected animals are kept in a basin with seawater and transported to the laboratory immediately.

Depuration of animals

The outer surface of the shell is scrubbed to remove epifauna and micro encrustations with a brush till the shell become clean. Animals are placed in a depuration tank of 75 l. capacity. The filtered seawater stored in a reservoir tank is passed through the UV chamber. The seawater from the reservoir tank to UV chamber passes through a quartz tube running in a zig-zag way, gets sterilized by UV irradiation. A continuous flow of UV sterilized seawater is arranged to the depuration tank from the U.V. sterilisation chamber. Animals are conditioned in the depuration tank with running UV treated seawater for three days. The water is fully aerated and the tank and the animals are cleaned every day (Fig. 1.2).

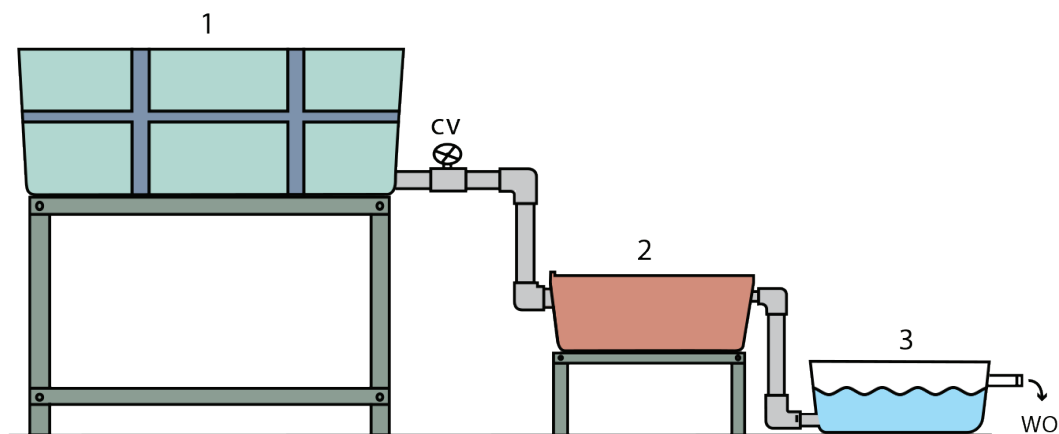


Fig.1.2. Diagrammatic set up of animal depuration system: 1. Water storage tank; 2. U.V. Chamber; 3. Animal depuration chamber; CV–Control Valve, WO – Water Outlet

Surface sterilisation of animals

The depurated animals are taken to the Tissue Culture laboratory in a sterile petriplate. The external surface of the shell is wiped with 70% ethyl alcohol soaked cotton and taken to the clean room aseptically.

Preparation of explant

The mantle tissue is excised off from the animals using a sterile knife. The central portion of mantle with more number of epithelial cells are taken by removing the outer edge of pallial organ and inner parts of connective tissue. The mantle strip is placed in Sterile SeaWater (SSW) in a petri plate and washed three or more times till the tissue is devoid of mucus and

other adherent particles. The strip is dipped in 35% alcohol for 15 seconds. After alcohol treatment, the strip is washed twice in antibiotic solution containing streptomycin 1000mg/ml and penicillin 2000 IU/ml for 10 minutes. Two final washings are given again in SSW to the strip and taken to the laminar flow hood by keeping in SSW in petri plates. The mantle strip is placed in a sterile glass plate and cut into 1 mm size of explant (Fig. 1.3).



Fig. 1.3 Preparation of explants

Inoculation and Incubation

The mantle explant is inoculated into a T25 flask/ petri plate by means of a sterile needle. After ensuring that the pieces are attached to the flask, 3 ml of culture media is added aseptically. The flasks are incubated at 28°C at pH 7.4 in CO₂ incubator. Osmolality of the culture media is maintained at 760 mOsm/kg (Fig. 1.4).

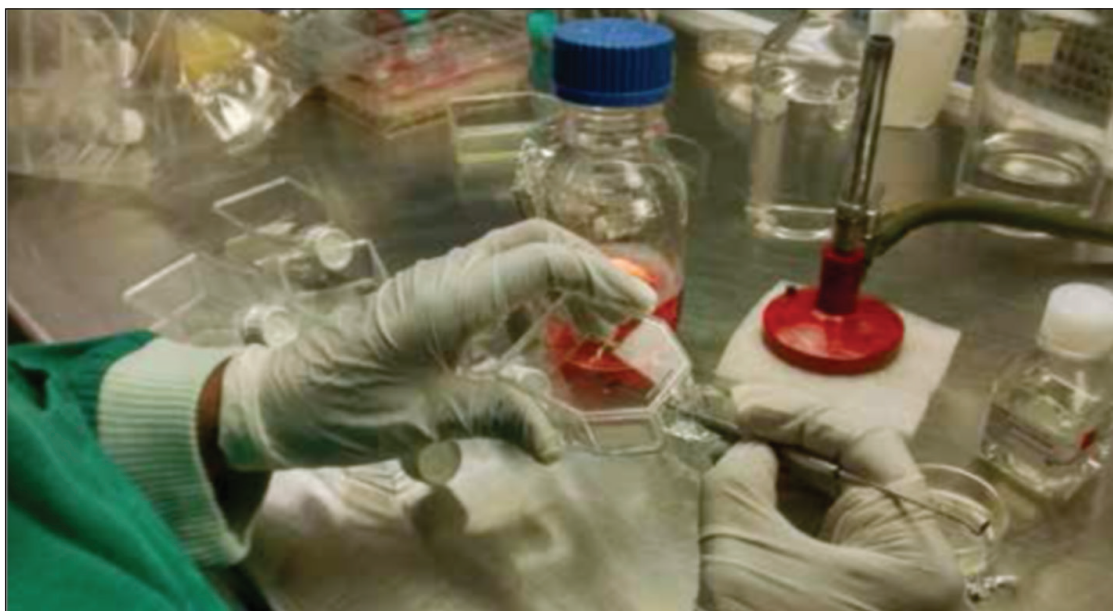


Fig. 1.4 Inoculation of explant

Observation and photography

The development of cultures is monitored regularly and the sequence of cell growth is photographed in the Phase Contrast Inverted Microscope-Nikon TMS with automatic exposure unit AFX-DX II.

Cell counting

Cells are counted in haemocytometer and viability is checked by trypan blue (0.4%) dye exclusion test. 0.1ml of 0.4% trypan blue stock solution is added to 0.9 ml of cell suspension and allowed to stand for 10 minutes at room temperature. The cell suspension is centrifuged for 5 minutes at 1000 rpm. The supernatant is removed and the cells are suspended in sterilized seawater (Hank's balanced salt solution). The stained and unstained cells are counted in haemocytometer (Susan Tolnai, 1975).

Analysis of crystals

The crystals formed in the *in vitro* and *in vivo* cultures are air-dried and are coated with gold by ion- sputtering (JEOL-JFC1200E), and observed by Scanning Electron Microscopy (SEM) and analyzed by an Energy Dispersive X-ray Spectroscopy (EDS).

Preparation of glassware, instruments and other materials

The setup of cultures need a meticulous and skilful approach. The processes described in the following steps are the general protocols to be followed in all the experiments.

Cleaning and packing

Glassware washing is a highly essential and time-consuming process. The disposable items such as syringes, bottles, and pipettes etc., supplied in sterile disposable packs are used whenever possible. The instruments and glassware, which are expensive and rather impossible to dispose of, are routinely washed and autoclaved. All the materials are soaked overnight in baths of 2% non-toxic detergent solution. Glassware is removed from the soaking baths and cotton wool plugs are removed from pipettes. All the glassware is washed at least six times with tap water and pipettes in automatic pipette washer. They are rinsed three times with deionised water and allowed to dry in air or hot air oven.

Pipettes are plugged with non-absorbent cotton wool placed in steel cans for sterilizing. The orifice of bottles, pipettes, measuring jars, screw caps, glass caps and rubber teats are covered with aluminium foil. The instruments like scissors, knife, forceps etc., are also covered with aluminium foil and placed in metal box for sterilizing. Petridishes are covered with butter paper using adhesive tapes.

Sterilization methods

The methods are followed as described by Adams (1980).

Hot air

This involves heating the glassware to 160°C for 90 to 120 minutes in an oven. It is a preferred method for glass bottles, pipettes, petri dishes, measuring jars and conical flasks.

Autoclaving

Heat stable solutions, rubber bungs, liners, bottles with plastic caps, ultra-filtration apparatus etc. are sterilized by steam treatment at elevated pressure of 15 lb for 20 minutes at 121°C in an autoclave. Empty bottles having plastic caps are loosely screwed on to allow penetration and escape of steam during the sterilization cycle.

Filtration apparatus is assembled with the filters in position and the whole apparatus is autoclaved. It is important to plug orifices with cotton wool and cover them with aluminium foil.

Filtration

Media containing plasma, serum, aminoacids or other materials are not autoclaved as they get denatured or spoiled at high temperature. The media are, therefore, prepared under strict sterile conditions. However, synthetic media and distilled water are sterilized by passing them through Millipore filters. The filters (0.22 µm) fixed in metallic holders, are fitted at the nozzle of hypodermic syringes or neck of large flasks. The filtration is done by passing the fluid to be sterilized through the Millipore filter under pressure. The pressure for filtration is created with a vacuum pump. For small quantities, syringe is used and pressure is exerted by the plunger. Filter sterilization also is done in sterile conditions of the culture room.

Storage of sterilized materials

The sterilized materials are stored in a pass box, which is provided with UV lamps. These materials are again sterilized with UV irradiation for 30 minutes before taken for culture work.

Preparation of solutions for tissue culture

Sterilized seawater (SSW)

Seawater collected from deep sea is filtered through Whatman filter paper; autoclaved at 121°C for 20 minutes and stored in glass bottles at room temperature.

Supplementary salt solution

102.4 g NaCl, 1.8 g KCl, 5.1 g CaCl₂, 11.8 g MgCl₂, 16.7 g MgSO₄ in 1 litre of double distilled water is autoclaved at 121°C for 20 minutes and stored in glass bottles at room temperature.

Lactalbumin hydrolysate (5%)

5 gm of lactalbumin hydrolysate dissolved in 100 ml of SSW is autoclaved at 115°C for 10 minutes and kept in glass bottles in refrigerator.

Nutrient medium

The culture media should be prepared in a perfectly sterile environment in the culture room. All the media are prepared in triple glass distilled water and made sterile by passing them through Millipore filters. But media containing proteins could not be filtered through Millipore filters and are prepared under strict sterile conditions. Media is prepared in triple distilled water with 2% M199, 25% supplementary salt solution, 5% Lactalbumin hydrolysate, 10% Foetal calf serum, and 0.1% antibiotic and stored in sterile bottles.

CELL CHARACTERISTICS IN EXPLANT CULTURES

C.P. Suja and S. Dharmaraj

Mantle tissue of the pearl oyster is responsible for the nacre secretion. Hence many workers started culturing the mantle tissue of molluscs to unravel the mechanism of calcification. Japan is the pioneer in developing tissue culture technology using marine organisms in 1970's. Samata *et al.* (1994) reported the SEM observation of microcrystals developed over black secretion on the cultured tissue of the pearl oyster, *P. fucata*. Belcher *et al.* (1996) and Falini *et al.* (1996) described the crystal nucleation in molluscs. In the research on mantle tissue culture of molluscs, many workers tried it in *in vitro* condition (Phuc *et al.* 2011; Barik *et al.* 2004; Awaji and Machii 2011). Kawai *et al.* (1981) first described the secretion of crystals in the explant culture of mantle tissue of pearl oyster. Suja and Dharmaraj, (2005) and Gong *et al.* (2008 a, b) achieved characterization of cultured crystals by *in vitro* culture of mantle tissue of the abalone and pearl oyster respectively. Marie *et al.* 2012 analysed the acid insoluble matrix of nacre and reported that thirty nacre specific shell matrix proteins control the microstructure of nacre. Matrix proteins control the crystal phase, shape, size, nucleation and growth of aragonite crystals (Liu *et al.* 2012; Xiang *et al.* 2013).

Cell proliferation from explant cultures

The cells start proliferating from the explant soon after incubation (Fig. 2.1). Cells of different morphological characters are migrated from the explant. The cells are found adhering to the culture plates and slowly move away from the explant. The cells after migration, start aggregating together and form groups. When the cells are initially liberated from the explant, they are mostly of round cells of both granular and agranular types. Dissociation of cells take place all around the explant and in few cases at a particular zone of the explant. Among the round cells, the granular cells are found more dominant than the agranular cells. The appearance of fibroblast cells is noticed rarely in day two and more commonly from day six to ten (Fig. 2.2). In some cultures, these cells appear late from 24 to 32 days. Some of the cells are not attached and seen suspended in the medium. These cells move to the periphery of the culture flask as the culture prolongs.

Some explant exhibit contractive movement in the culture medium and the same continues upto six to eighteen days. The movement indicates the viability of the explant. This explant produce round epithelial-like cells in large numbers.



Fig. 2.1 Cell proliferation from explant culture

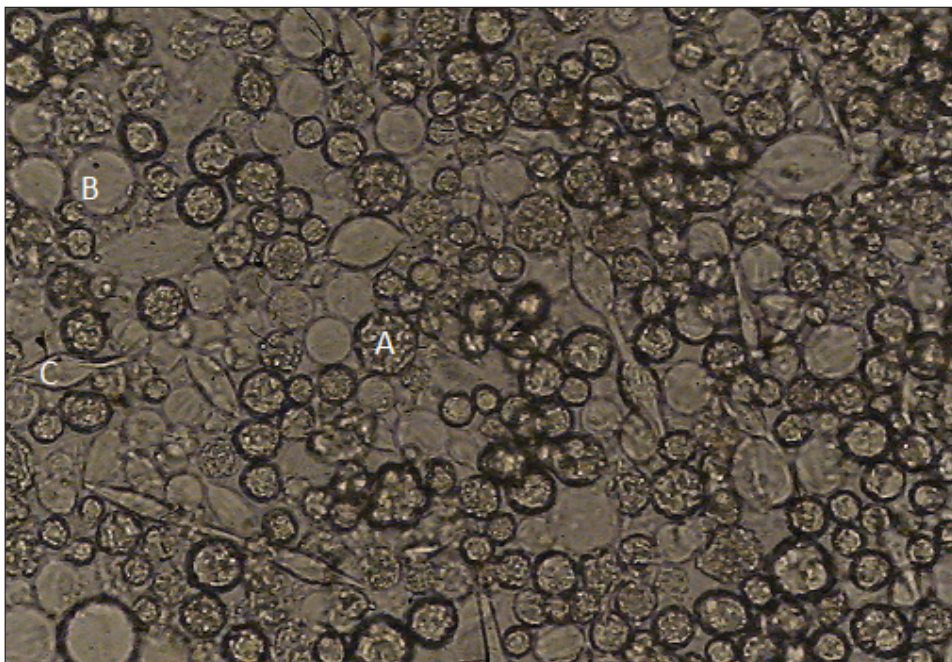


Fig. 2.2 Different types of cells: A) round cells with organic granules (granular cells);
B) round cells without organic granules (agranular cells); C) fibroblast cells

The round granulated cells dissociate from the explant, develop pseudopodia in all directions and later form a pseudopodial network. The pseudopodia disappear after five or six days and reappear in another place, most often in a circular pattern. Pseudopodial network remain in the plate upto twenty-five days. The round cells with organic granules become larger over the thick pseudopodial network, initiate the crystal formation (Fig. 2.3).

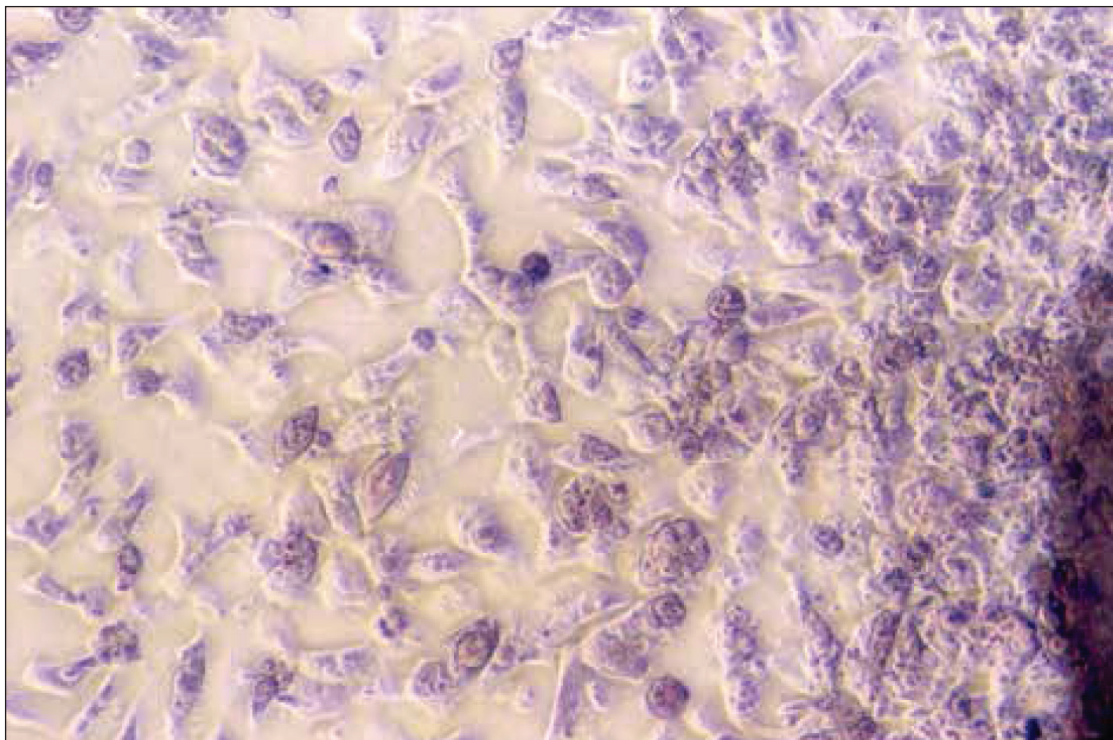


Fig. 2.3 Granulated round cells over the pseudopodial network

The granulocytes turn to dark brown and develop an epitheloid cellular sheet. The granulocytes are not perfectly round but have uneven cell boundary with pigmented appearance. A granulocyte seems to contain about three to twenty two granules depending on the size of the cell. At this stage, the granules exhibit different colours that are similar to nacre colour. Some of the granules observed are pink or green in colour (Fig. 2.4). The organic granules grow larger and liberate out in the form of individual crystals. These crystals on liberation, exhibit reflection under phase contrast microscope. The crystals grow further to large sizes. The crystals thus liberated, aggregate together to form a nacreous layer by binding with inter lamellar matrix.

Rhombohedral shaped crystals are also observed in some cultures. These are seen individually in groups inside the medium as well as over the explant.

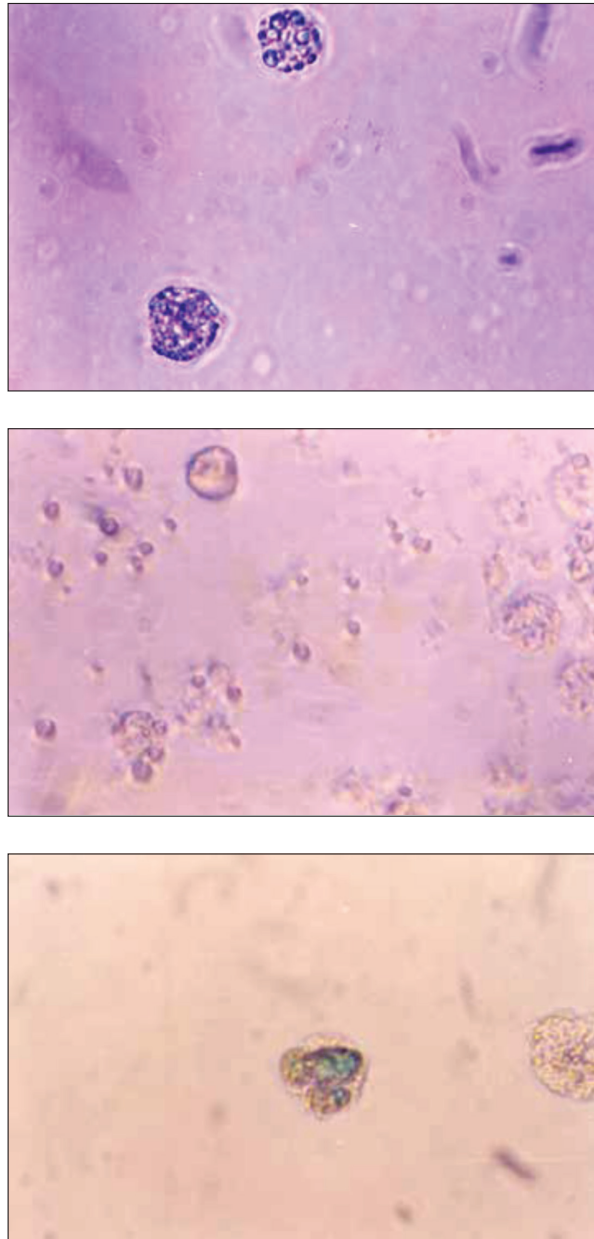


Fig. 2.4 Cells liberated organic granules with different colours (Suja and Dharmaraj 2005)

Cultures obtained from a healthy animal can be prolonged more than one month without the renewal of culture medium. Mitotic activity is occasionally observed in some cultures. Subcultures have been done three to six times with the first passage at an interval of three or four weeks and later from six to eight weeks at a concentration of 2×10^4 cells/ml. Mostly, cultures have a maximum life span of 370 days.

During the course of the experiment, it is found that about 30% of cultures of mantle explant are interrupted due to microbial contamination. Filamentous fungal contamination is also encountered with rare occurrence of bacteria and contaminated cultures are discarded immediately (Fig. 2.5).



Fig. 2.5 Microbial contamination: A) fungus and B) Bacteria

A rainbow tinted nacre-like substance is observed in culture plates after 24 hours of culture initiation, which resembles the nacre colour of the shell. The nacre-like substances continue to be present throughout the culture period. Some crystals are seen over the explant. It is observed that the growth of cells is better in T 25 flasks than in petri plates.

Previous studies have shown that primary explant cultures from the mantle are suitable *in vitro* models to study the process of biomineralization, maintaining cell viability and cell-to-cell interactions (Kleinschuster *et al.* 1996). Machii and Wada (1989) also reported the strong calcium peaks in *in vitro* crystals in the explant cultures of *Pinctada* with high calcium content optically showing strong birefringence. Samata *et al.* (1994) reported the SEM observation and EDS analysis of crystals over the black secretion and found that these types of crystals are rich in calcium. This study gives light to the basic understanding of cell proliferation and crystal formation in *in vitro* conditions.

Chapter 3

EXPERIMENTS WITH THE MANTLE TISSUE OF DIFFERENT PEARL PRODUCING MOLLUSCS

C.P. Suja and S. Dharmaraj

Different pearl producing molluscs have been selected for the experiments. The mantle tissue which is responsible for the pearl or nacre formation is excised off in sterile condition from the animal and utilized for culture. This tissue is again cut into small pieces called explant for inoculating in the media.

Many research studies have been done in the aspect of different media used for the explant culture of marine mollusc tissues. Tripp (1963) reported the medium consisting of animal sera and seawater for the oyster heart explant culture. The oyster cells could be maintained up to 42 days in a medium containing various oyster extracts was established by Perkins and Menzel (1964). Machii *et al.* (1985a) and Machii and Wada (1989) used a medium Pf-35 for the explant culture of the tissue of *P. margaritifera*, *P. fucata* and other marine invertebrates. Li *et al.* (1966) used M 199 with different additions for the culture of cardiac tissue cells of the oyster, *C. virginica*. Cecil *et al.* (1976) studied the property of extracts from echinoderms on cell cultures of echinoderms and molluscs. For *in vitro* maintenance of amoebocytes from the American oyster, Brewster and Nicholson (1979) used a medium prepared with marine saline and supplemented with fetal bovine serum and other supplements for *C. virginica*. Machii *et al.* (1985b) selected Medium Hd-1 for the organization of a cell line from abalone, *Haliotis discus*.

Development of an ideal culture medium is important, to enhance the cell adherence, cell yield and most importantly, for the formation of nacreous layer.

Haliotis varia

Cell proliferation from the explant culture of mantle tissue of the abalone, *H. varia* is recorded every day. The number of cells proliferated is plotted against the days of culture. Value of each point represents the average of eighteen replicates. A progressive trend is observed in cell proliferation from day one to four and it varies from experiment to experiment. On day one, the average cell concentration is 7×10^4 cells/ml in the first experiment and it is 6×10^4 cells/ml in the second experiment. On day four, it is around 11×10^4 cells/ml and 9×10^4 cells/ml, respectively. Crystal initiation is noticed on day eight onwards and its appearance varies depends on the viability of cells (Fig. 3.1 and 3.2).

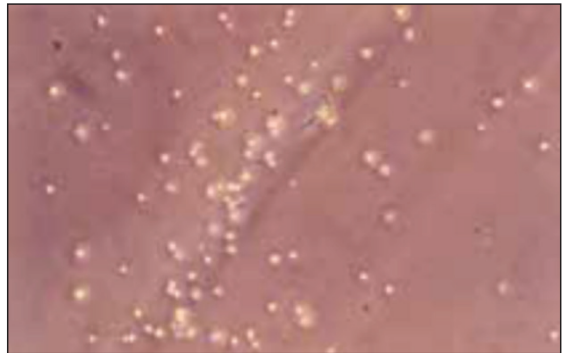
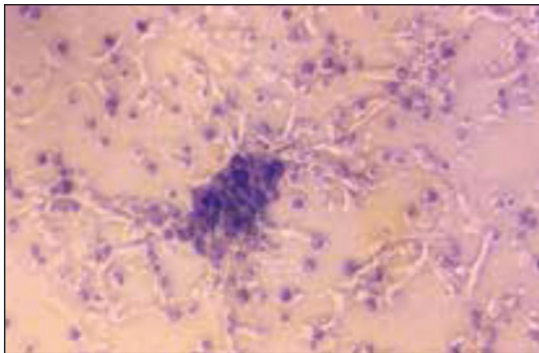


Fig. 3.1 Cell proliferation and crystal initiation from the explant culture of mantle tissue of the abalone, *H. varia* (Suja and Dharmaraj, 2005)

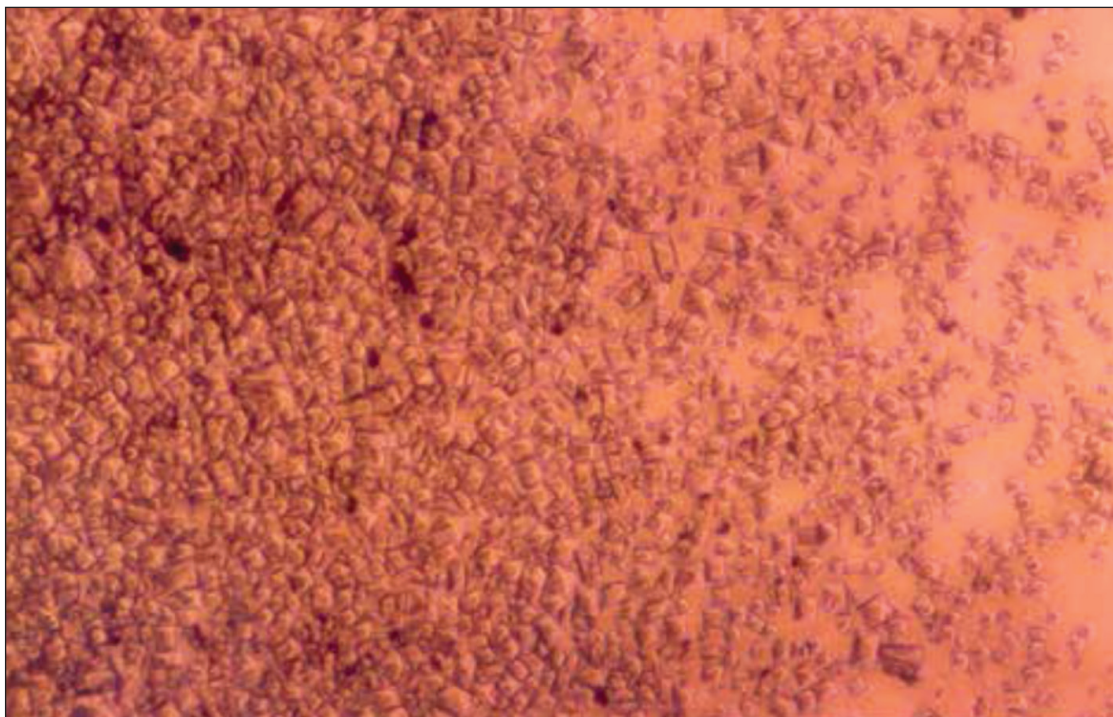


Fig. 3.2. Crystal formation achieved in *H. varia*

The type of cells dissociating from the explant differs from explant to explant and experiment- to-experiment. The size of each type of cells varied. In most of the cases, the cells liberated are of round and spherical shape along with stray occurrence of fibroblast-like cells. The size of round cells varies from 5 to 25 μm with an average value of 16.1 μm . The round cells are of two types i) granulocyte or granular leucocyte and ii) agranulocyte or lymphocyte or hyaline leucocyte. The round cells are the dominant cell type at early stages of culture. Subsequently smaller epithelial-like cells and spindle shaped fibroblast-like cells appear. The fibroblast-like cells are highly transparent. The size and shape of these cells varied markedly at various times in different cultures. In some explant cultures, the fibroblast-like cells are stumpy in shape having a minimum of 35 μm and a maximum of 120 μm (average: 97 μm). The width of these cells ranged from 3 to 20 μm with an average of 14 μm (Fig. 3.3). The granulated cells have liberated the crystals (Fig. 3.4).

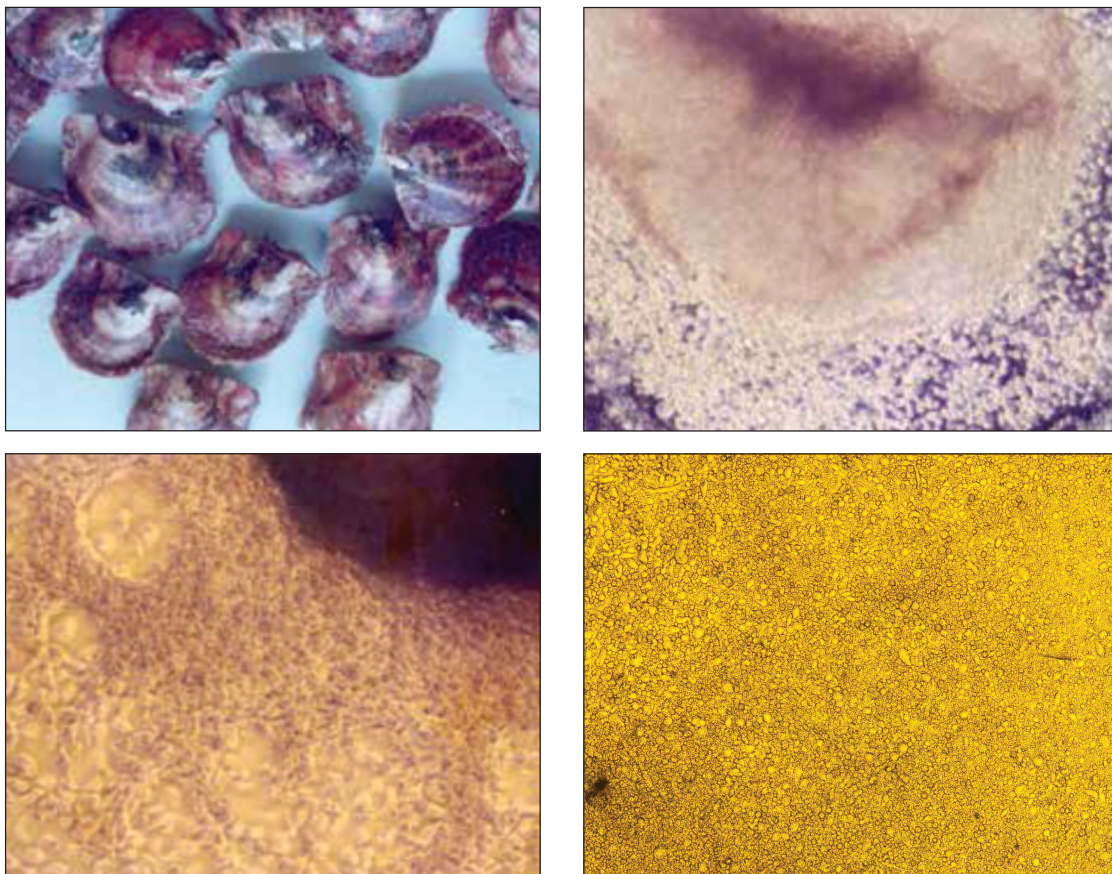


Fig. 3.3 Cell proliferation from the explant culture of mantle tissue of the *P. fucata*

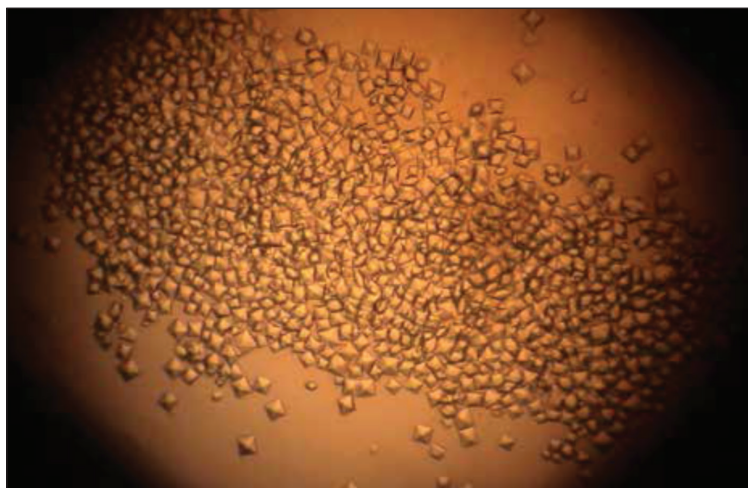


Fig. 3.4 Crystal formation achieved in *P. fucata*

Pteria avicular

Experiments of explant and organ cultures are organized with the mantle tissue of *Pteria avicular*. Small round cells of size 2 to 5 μm are liberated from explant cultures. Cultures are kept for thirty days and initiation of crystals noticed (Fig. 3.5).

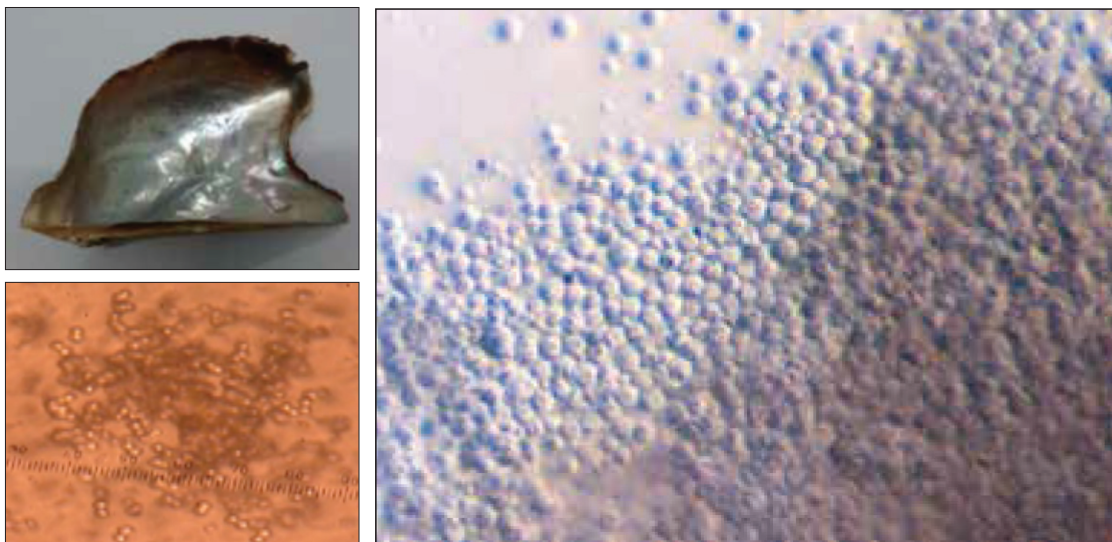


Fig. 3.5 Cell proliferation and initiation of crystal formation from the explant culture of mantle tissue in *P. avicular*

Further experiments of organ cultures are conducted with different types of materials like glass, teflon and silica. Cells are proliferated and formed organic matrix and pearl sac around all the types of materials used in cultures. Good number of crystals are developed over all the materials in culture plate.

Perna viridis

In vitro explant cultures by using mantle tissue of green mussel, *Perna viridis* have also been conducted. Good number of cells are proliferated with a size range of 5 to 10 μm and with an average size of 6 μm in the explant cultures (Fig. 3.6). Nacre crystals are observed in the culture plates after 60 days and joined to form a layer. Organic matrix, pearl sac and crystals are developed in organ cultures around the nucleus.

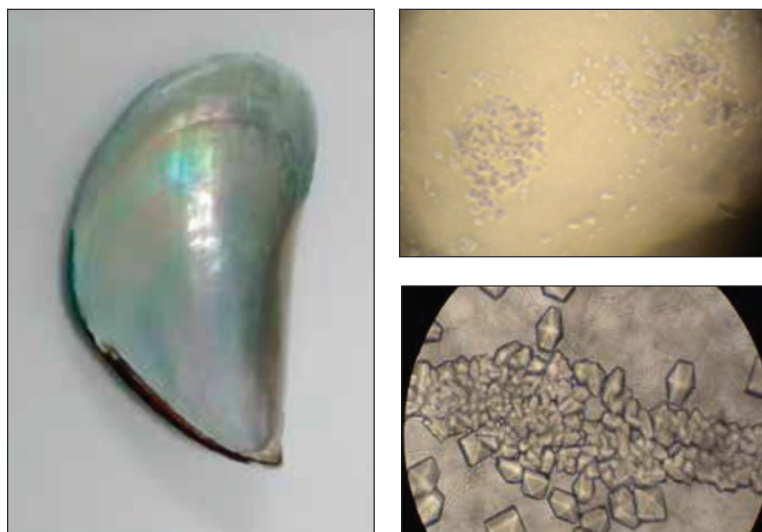


Fig. 3.6 Cell proliferation and crystal formation from the explant culture of mantle tissue of *P. viridis*

Placuna placenta

Explant and organ cultures are conducted with the mantle tissue of window pane oyster, *Placuna placenta*. Good numbers of cells are developed in the explant cultures. Granular cells 5 to 15 μm and agranular cells of 10 to 25 μm size are noticed with an average size of 10.5 and 19 μm respectively. Fibroblast cells of different size and shapes of 70 to 300 μm with an average 130 μm recorded after thirty days. Crystals are also recorded after seventy five days and the cultures are maintained up to ninety days (Fig. 3.7). Organ cultures are also carried out and formed pearl sac.

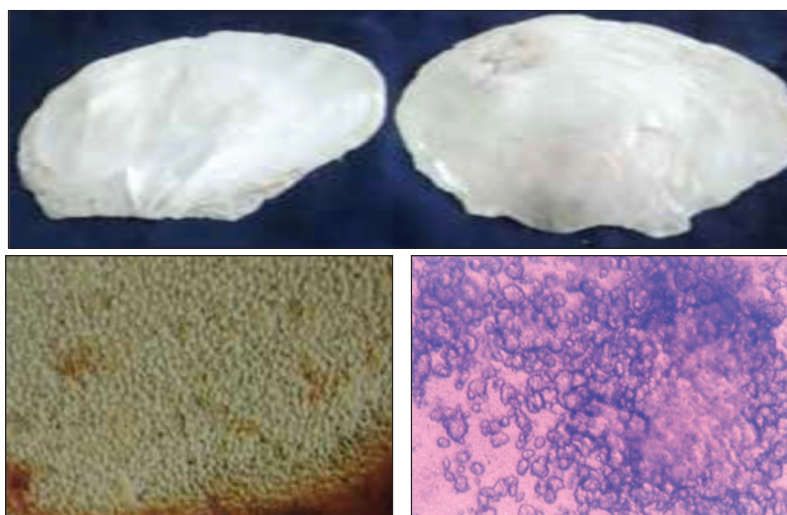


Fig. 3.7 Cell proliferation and initiation of crystals from the explant culture of mantle tissue of *P. placenta*

Atrina vexillum

Cell characteristics are studied by the explant culture of the mantle tissue and the cell size is recorded. Granular cells of 3 to 20 μm size and agranular cells of 10 to 20 μm size are released from the explant cultures (Fig. 3.8). Crystals are developed after 20 days. In the organ cultures, cells proliferate over the nucleus and forms a pearl sac.

Pinna bicolor

In the mantle explant culture, cell size is noticed. Granular cells of 2 to 10 μm size and agranular cells of 15 to 22 μm size are released from the explant cultures (Fig. 3.9). In the organ cultures, cells grow over the nucleus and a pearl sac is formed.



Fig. 3.8 Cell proliferation from the explant culture of mantle tissue of the *A. vexillum*

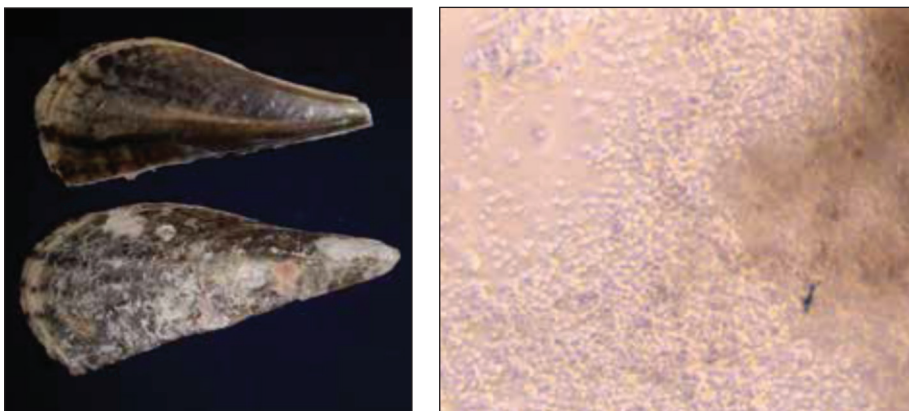


Fig. 3.9 Cell proliferation from the explant culture of mantle tissue of the *P. bicolor*

Modiolus traillii

Two experiments have been conducted in the explant and organ culture of *Modiolus traillii*. Cell characteristics are studied in the explant cultures. Granular cells of 3 to 10 μm size and agranular cells of 10 to 20 μm size are released from the explant. Fibroblast cells are noticed on third day. In the organ cultures, cells grow over the bead. Crystals are developed after one month. Cultures have been maintained up to 50 days (Fig. 3.10).



Fig. 3.10 Cell proliferation from the explant culture of mantle tissue of the *M. traillii*

Table 3.1. Cell Characteristics of different pearl producing molluscs

Animal	Cell Type	Cell size μm
<i>M. traillii</i>	Granular	3-10
	Agranular	10-20
<i>A. vexillum</i>	Granular	3-20
	Agranular	10-20
<i>P. bicolor</i>	Granular	2-10
	Agranular	15-22
<i>P. avicular</i>	Granular	2-5
<i>P. viridis</i>	Granular	2-15
<i>P. placenta</i>	Granular	5-15
	Agranular	10-25
<i>H. varia</i>	Granular	3-16
	Agranular	13-18
<i>P. fucata</i>	Granular	5 -25
	Agranular	10-22

Studies on the effect of contaminant microorganisms with cultures

Unusual over growth of crystals are noticed in some contaminated cultures of *P. fucata* within a limited period of one week. Contaminants are isolated and identified as three fungi and one bacterium viz. *Aspergillus* sp. and *Brevundimonas* sp. (Fig. 3.11). The contaminant microorganisms are inoculated into the growing cultures to find out the possible influence on biomineralizations. Mass growth of nacre crystals are observed in culture plates in a lesser time (Fig. 3.12).

Study with different areas of mantle

Two experiments of explant cultures have been conducted with different portions of mantle tissue of *P. fucata*. Even though the cells are released from all the explant, it is observed that, the explants taken out from the lower portion of mantle tissue with fat cells are easily contaminated than the upper mantle region.

Study with different culture vessels

Experiments of mantle organ culture have been conducted in T25 flasks and culture tubes to know the difference in growth. In both these vessels, cells are proliferated around the bead and form pearl sacs. Crystals are also developed. Cell growth can be observed in T25 flask where as it is difficult to observe the growth in culture tubes. Culture media usage and area can be minimized in culture tubes (Fig. 3.13).

Study with different number of explants

Experiments are conducted with different number of mantle explants of *P. fucata* to minimize the period of pearl sac formation. One to three numbers of explants have been used for a single nucleus to know the period of growth. It is found that, two or three explants will form a pearl sac more quickly than a single explant. However if one gets contaminated it will adversely affect the other explant and the pearl sac formation.

Experiments with round and flat beads

All the previous experiments are conducted on round nucleus for the growth of cells and nacre formation. The measurement of thickness of nacre growth is difficult to find out on round beads. Hence experiments are conducted on half beads. The cultures with explants on hemispherical beads also developed pearl sacs.



Fig. 3.11 Isolated contaminants in culture tube



Fig. 3.12 Morphologically complex doughnut-shaped structures consisting of densely packed layers of plate like aragonite crystals noticed under SEM in fungus contaminated beads



Fig. 3.13 Culture maintaining in T25 flasks and culture tubes

Experiments with different materials as nucleus

Experiments of organ cultures are conducted with different types of materials such as glass, teflon and silica apart from shell bead. Cells grow and proliferate around all the types of materials used. Good number of crystals are developed over all the materials in culture plate (Fig. 3.14 and 3.15). However, good adherence and prominent mineralization is observed over shell bead. Raman spectra also confirm the same.



Fig. 3.14 Crystals over glass bead with the mantle tissue of *P. fucata*

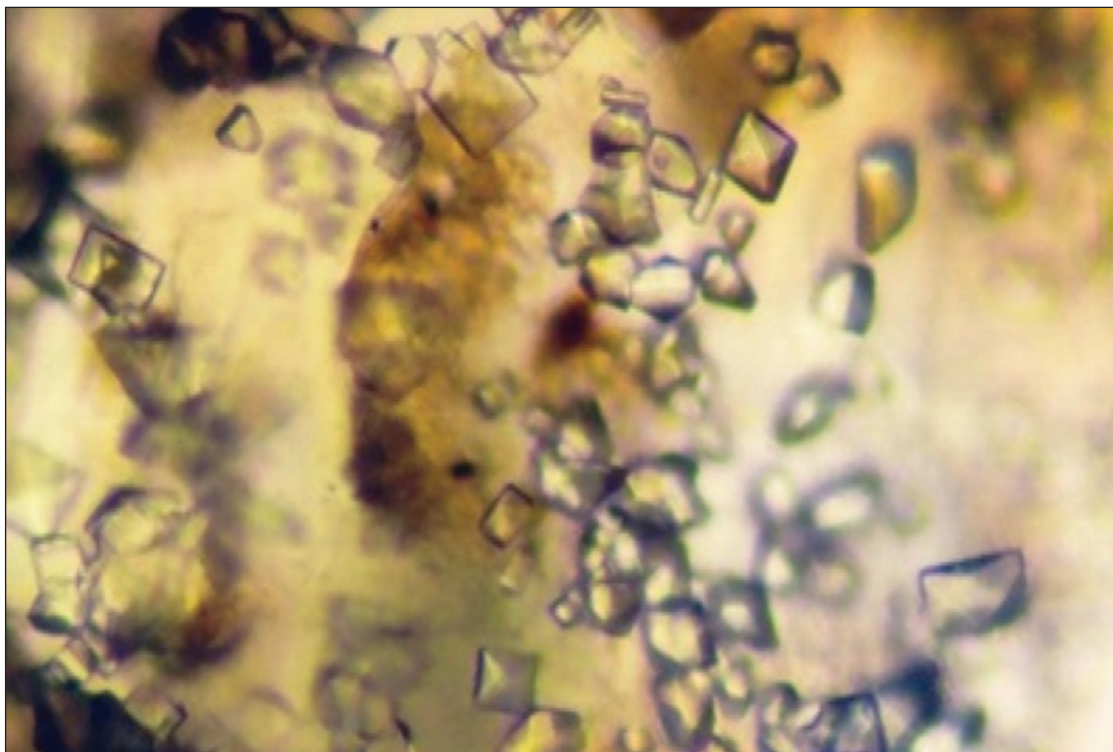


Fig. 3.15 Crystals developed over a silica bead

Cell proliferation of mantle tissue of *P. fucata* in various media

Media preparation

Media is formulated with supplements from marine source.

Extraction of Water Soluble Matrix (WSM) from nacre

Shell of *P. fucata* is treated with dilute Hydrochloric acid and the nacre part of the shell is separated. The nacre is crushed and ground into a powder with mortar and pestle. Two grams of this powder is dissolved in 200ml phosphate buffer solution (PBS). The solution is stirred overnight at 4°C and then centrifuged at 16000rpm. The supernatant is freeze dried in a lyophilizer at -55°C for 5 hours to obtain the WSM in powdered form.

Preparation of microalgal powder

Microalgae, *Isochrysis galbana* is harvested from the mass culture and centrifuged at 10000 rpm at 22°C for 10minutes. The biomass is collected and freeze dried in a lyophilizer at -55°C for 3 hours to get it in powder form. This powder is stored in the refrigerator in sealed covers and is used for incorporating into the medium.

Body fluid (BF) extraction

Body fluid of the pearl oyster *P. fucata* is taken out with a syringe from the space between the shell and the mantle. This suspension is filtered through 0.22 μ filter and stored for the incorporation in media.

Preparation of different culture media for organ culture

The seawater basal medium is prepared by dissolving 0.8 g of Agar in 100 ml of seawater and autoclaving at 121°C for 20 minutes. Supplements are added as in Table 3.2 and used for culture by pouring 5ml each into the culture flasks with 0.001 g/ml of kanamycin.

Table 3. 2 Composition of media for *in vitro* nacre layer characterization of pearl oyster

Medium	Components
1	Sterile Sea Water (SSW)
2	SSW + 0.1% <i>I. galbana</i> powder (ISO)
3	SSW + 0.1% Water Soluble Matrix (WSM) of <i>P. fucata</i>
4	SSW + 0.1% WSM + 0.1% ISO.

Inoculation of mantle tissue

The mantle tissue of *P. fucata* is taken out, given antibiotic treatment and inoculated in respective media for cell characteristic studies. Cell proliferation is assessed by counting the cells in explant cultures after eight days. Explants are prepared and inoculated in tissue culture plates over the bead in different formulated media for nacre characterisation.

Cell proliferation

After 24hrs of initiation of culture, cells start proliferating from the explant in the different media used for experiment. The cells are of different morphological types such as hyalinocytes, granulocytes and fibroblast-like cells. The cells migrate away from the explant and adhere by developing pseudopodia to form a network like arrangement covering a large area in subsequent days. In the culture with WSM medium, cell proliferation is minimal but a matrix like formation is visible surrounding the explant. The cell suspension is taken after eight days, the cells are counted in haemocytometer and tabulated in Table 3.3. Maximum cell liberation is noted in media supplemented with *I. galbana* powder and WSM. It is noted that maximum number of cells of 45.69×10^4 cells/ml are released in the combination of ISO and WSM with sea water followed by 41.13×10^4 cells/ml with a combination of ISO, WSM

and M199. There is not much difference of cell liberation in cultures with SSW, SSW+ISO and SSW+BF.

Table. 3.3. Cell proliferation in different media in the explant culture of *P. fucata*

No	Media	Cell x10 ⁴ cells/ml
1.	SSW	24.69
2.	SSW+ISO	24.13
3.	SSW+WSM	7.33
4	SSW+M199	18.19
5.	SSW+ISO+ M199	34.13
6.	SSW+WSM+ M199	12.69
7.	SSW+ISO+WSM	45.69
8.	SSW+ISO+WSM+ M199	41.13
9.	SSW+BF	23.21

Characterization of nacre layer formation

Scanning Electron Microscopy (SEM)

Various stages of coatings are analysed on beads in media compositions of SSW, SSW+ ISO, SSW+WSM and SSW+ISO+WSM after 30 days. Nacre coating by merging and fusion of individual crystals is observed and its transformation into the nacre tablet has been clearly visible over the bead in the medium with ISO in SSW (Fig. 3.16 A and B).

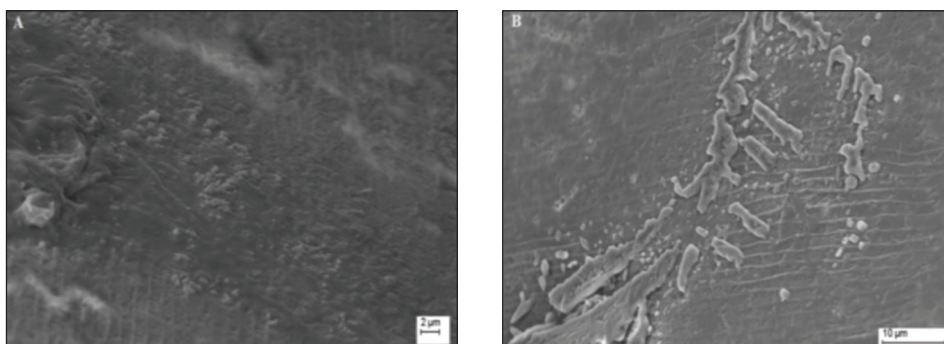


Fig. 3.16 Deposition of nacre crystals and its transformation over the bead in SSW with ISO

Disordered crystal deposits are noticed over the bead in SSW without any supplements (Fig. 3.17A and B). In the culture medium incorporated with WSM, orientation of crystals is observed uniformly over the bead (Fig. 3.18. A and B). Nanocrystals in nacre layer have been noticed over the bead in medium constituted by WSM and ISO in SSW (Fig. 3.19. A and B).

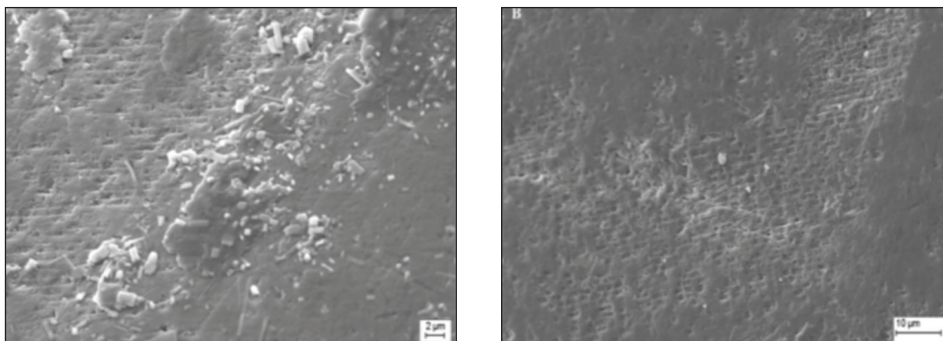


Fig. 3.17 Disordered crystal deposits over the bead in SSW alone

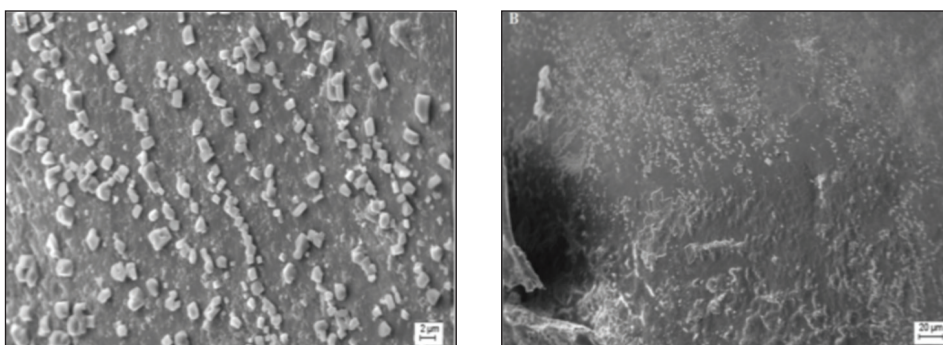


Fig. 3.18 Early stage crystal formation over the bead in SSW with WSM

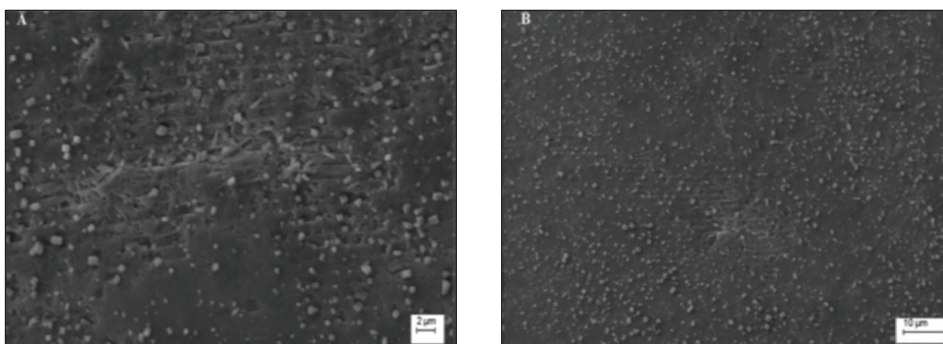


Fig. 3.19 Nanocrystals in nacre layer over the bead in SSW with ISO and WSM

Energy Dispersive X-ray Spectroscopy (EDS)

The EDS report of control and experimental shell beads with different media is shown in Table 3.4. Biomineralization of nacre is found to be higher in the medium formulated by ISO in SSW. The calcium (Ca) percentage of nacre coating on beads in the medium SSW with ISO is 32.33 ± 0.26 which is comparatively higher than control beads and other beads in different media. The combination of WSM and ISO in SSW also induced the CaCO_3 deposition over the bead.

Table 3.4. Report of EDS with different bead maintained in various media

Media	Ca (%)
Control Bead	22.75
SSW	27.30
SSW+ISO	32.33
SSW+WSM	22.90
SSW+WSM+ISO	27.68

SSW-Sea Water, ISO-Lyophilized *I. galbana* powder, WSM-Water Soluble Matrix from *P. fucata*.

In this present work on cell proliferation, its characteristics and crystal formation in mantle explant cultures of different molluscs *P. fucata*, *P. avicular*, *A. vexillum*, *P. bicolor*, *M. trallii*, *P. viridis* and *P. placenta* are studied and compared with the result of *H. varia* reported by Suja and Dharmaraj (2005). Group of rhombohedral crystals are noticed in *P. fucata* and *P. viridis* as in *H. varia*. Daugavet and Blinova (2015) reported rhombohedral crystals in mantle cultures of *Mytilus edulis* after four months. This work justifies the report of Wada (1985) that the small spherulite of calcium carbonate grew after four days of operation. In this work, microbial contaminants are also identified. Suja *et al.* (2014) studied the effect of antimicrobial agents in cell proliferation of mantle tissue of *H. varia*. Suja *et al.* (2017) reported the effect of different media in the cell proliferation of *P. viridis*. In this present report, cell proliferation and characteristics are studied with different species with Medium 199.

Calcium is detected from all the crystals examined. Morphology and size of the crystals varied in different cultures and in the same culture. Wada (1961) pointed out that the shell substances are crystallized out after passing through the amorphous state when the solid phase separates from the liquid phases. Amorphous crystal formation is noticed in the present work in *P. placenta* and *P. avicular*. In the explant cultures, the free crystals could

not be collected for the analysis as most of them are attached to the plate. However, by *in-situ* examination, the free crystals show high birefringence under polarized light and are hexagonal in shape. Wada (1961) reported that the twin of aragonite exhibited regular hexagonal form, which consisted of three grains joining together on its twinning face. It exhibited the twinning types of aragonite at all times during growth of the nacre in pearl oyster shell.

Both calcite and aragonite crystals are seen in the same culture flask in the present work in some experiments. The rhombohedral crystals show high birefringence. Under polarized microscope, aragonite nature is clearly reflected. Aragonite, under ordinary conditions of temperature and pressure, is relatively unstable and changes to calcite, although the rate of change is very slow. Crystals of aragonite have been observed which have completely changed to its stable polymorph calcite (Ford, 1932). Wada (1961) stated that the fixation of calcium and the orientation of mineral crystals are governed directly or indirectly by the elongation of epithelial cells of mantle tissue in shells and the crystals grown freely from each other exhibits rhombic, irregular or circular shape. In the present study, the cultured cells of mantle of abalone secrete oolite, hexagonal and rhombohedral crystals of calcium carbonate with extreme birefringence showing their calcite and aragonite nature.

By these studies, it is proved that the mantle cells of pearl producing molluscs are capable of secreting calcium carbonate crystals of high birefringence in *in vitro* conditions. The possibility of *in vitro* biomineralization in sea water supplemented with algal powder is proved in *P. fucata*. More studies have to be done in this line to standardize the correct dosage of supplements like WSM and microalgal powder to get more deposition within limited period. The role played by the microenvironment on the formation of aragonite and calcite crystals needs further investigations. The crystals developed in the *in vitro* cultures are the secretions from the mantle tissue of molluscs. It is essential to study the interventions of various supplements of nutrient media and stimulation of cell activity for biomineralization.

ORGAN CULTURE OF MANTLE TISSUE OF DIFFERENT PEARL PRODUCING MOLLUSCS

C.P. Suja and S. Lakshmana Senthil

Organ culture of mantle tissue improves the understanding of early pearl sac formation. The mechanism of calcification in marine molluscs was studied by the culture of mantle for many years (Suja and Dharmaraj, 2005; Gardner *et al.* 2011). Biomineralization is a process of hard tissue formation by living organisms. Characterization of biomineralization is a highly controlled and functional process (Liang *et al.* 2014). It forms inner aragonite nacreous and outer calcite prismatic layers (Inoue *et al.* 2011).

Study of pearl sac development through organ culture

Media (M199) mixed in agar is used for seeding the nucleus or shell beads. The nucleus or beads are also sterilized before inoculation. Explant is inoculated on one side of the bead in a culture vessel. Cells proliferate from the explants from day one onwards. The proliferated cells spread around and over the bead and a pearl sac of epithelial cells is formed. The semi solid medium provides a better substratum for holding the bead to keep it stationery and facilitates the growth of explants over it. Thick mass of cells are seen from all sides of the bead as pointed structures which is an indication of spreading of cells (Fig. 4.1). The cells start secreting crystals and a nacreous layer is formed over the bead. The crystals are analyzed through SEM and EDS and presence of CaCO_3 crystals are proved in pearl oyster and abalone at different periods. A complete lustrous pearl can be formed through more layers of coating over the bead. Similar experiments are conducted in other molluscs like *P. avicular*, *P. viridis* and *P. placenta*. The formation of pearl sac and the formation of crystals are noticed over the bead in these molluscs also. The present study is taken up to develop the technology on *in vitro* pearl formation by understanding the aspects of cell behavior in pearl sac formation. The investigation will serve as a stepping stone for advanced research in *in vitro* pearl production in all molluscs and thus can revive the pearl culture.

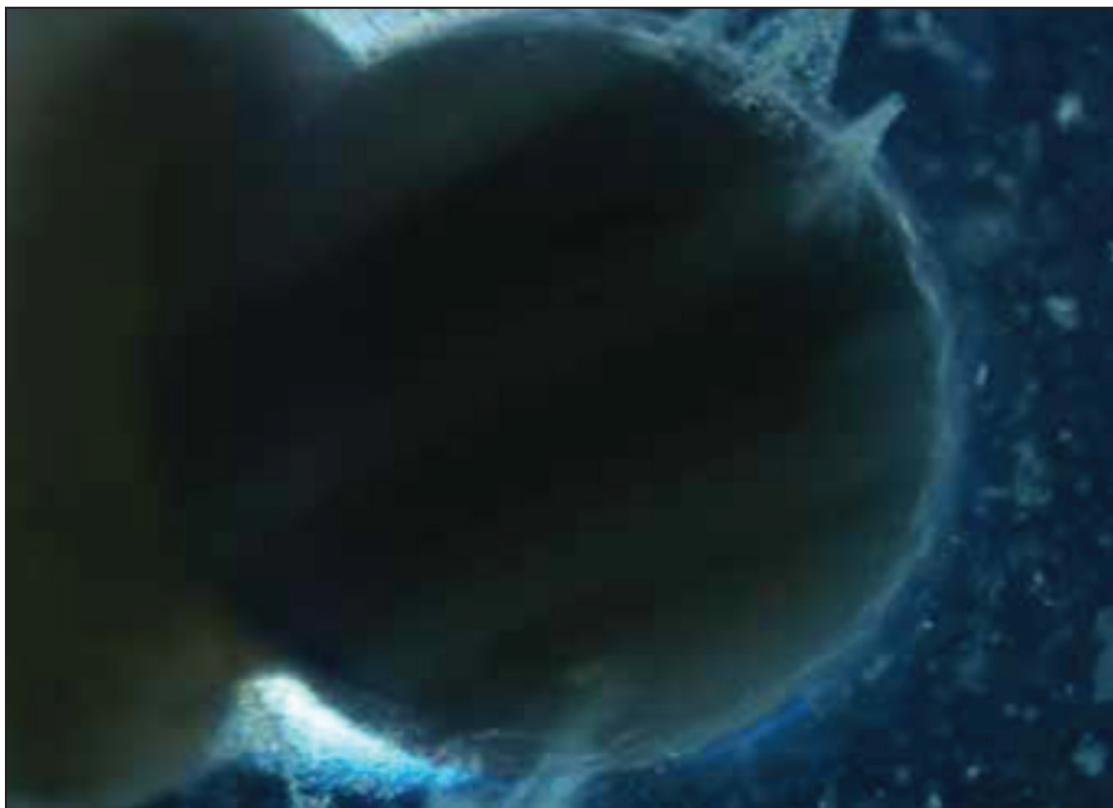


Fig. 4.1. Explant releasing cells around the nucleus to form pearl sac

Cells from explant cultures can also be used for pearl sac development. Cells released from one to two month old explant cultures are taken aseptically and inoculated over the nucleus. Cells grow over the nucleus and develop the pearl sac. Later, crystals are developed over the bead. These experiments have been carried out since 2008 (CMFRI Ann. rep. 2008-09)

Achievement of nacre layer formation in three different pearl producing molluscs

Formation of pearl sac

The pearl sac formation by using a small piece of mantle tissue of the pearl producing oysters is shown in Figure 4.2. The round epithelial cells proliferate from the mantle tissue and spread over the shell bead. Accumulation of such cells on the shell bead result in the formation of pearl sac. Later the cells of the pearl sac produce organic matrix. The organic matrix induces the cells to secrete crystals and deposit on the bead. The crystals develop from the sub microscopic nuclei by a process known as crystal nucleation. However, calcium carbonate coating and nacre layer formation over the bead vary depending on the species.

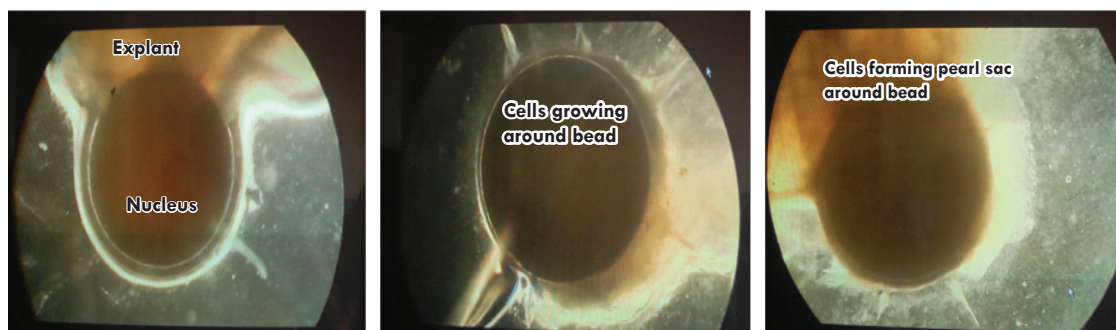


Fig. 4.2 Microscopic observation of different stages of pearl sac formation

In vitro* organ culture by using mantle tissue of *H. varia

The crystals developed in the *in vitro* cultures are the secretion of mantle tissue of *H. varia* and the report of crystal from the *H. varia* explant and organ culture is the first of its kind. Having achieved a success in the basic technology in *in vitro* pearl formation where the formation of nacreous layer is demonstrated, further experiments are conducted to study whether the nacre growth is continuous throughout the culture period or the growth is ceased at a particular period of time in *in vitro* conditions. Deposition of more crystals on the bead can result in the formation of multiple nacre layers to form an *in vitro* pearl.

Characterization of nacre layer

Scanning Electron Microscopy

The attachment of mantle tissue of *H. varia* to the shell bead is shown in Fig. 4.3. Nacre is produced within fifteen days of initiation of cultures. The experimental beads are analysed after ninety days. The organic matrix provides nucleation sites for crystal formation and influences the type of crystals to be produced. In organ cultures, crystals are seen embedded in the organic matrix scattered over the bead (Fig. 4.4).

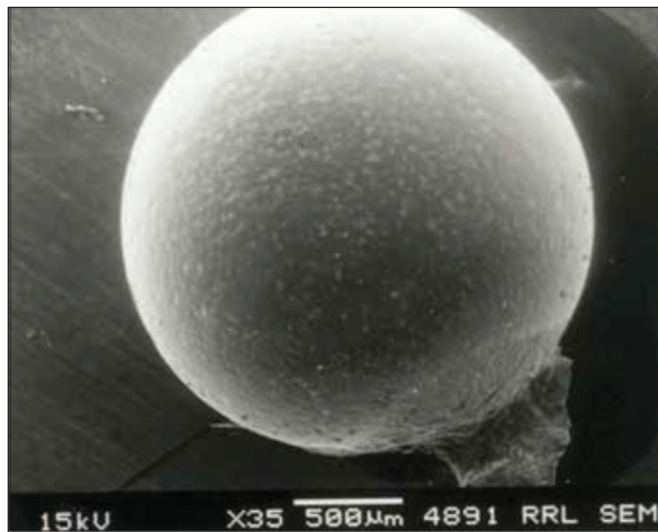


Fig.4.3 SEM image of attachment of mantle tissue of *H. varia* to the shell bead

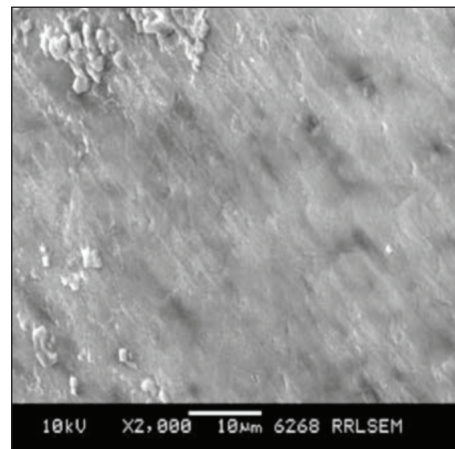
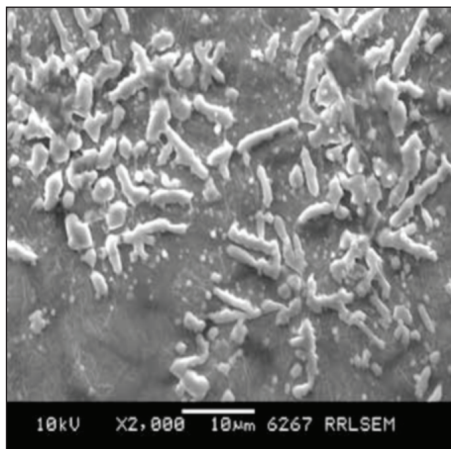


Fig. 4.4 SEM images of surface of the experimental bead in 90 days

Crystals scattered on the surface of the bead increase in size, come into contact with one another and finally develop into a thin crystal lamella. This scattered crystals and its linking are visible in the analysis of experimental beads in 90 days.

An organic membrane is sandwiched, when a crystal joins with adjacent ones and is found as a groove on the surface. New crystals are deposited over the lamellar layer. The SEM images of the growth of crystals in 180 days, 270 days and 365 days are given in Fig. 4. 5, Fig. 4. 6 and Fig. 4. 7 respectively.

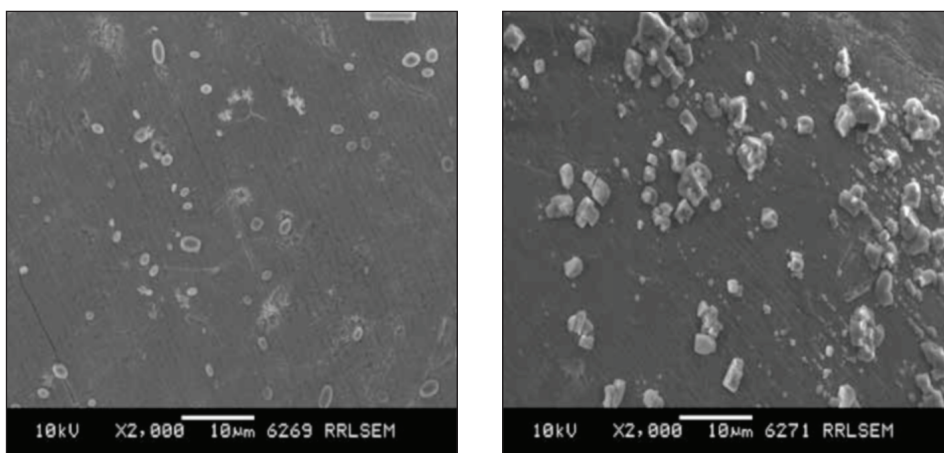


Fig. 4.5 SEM images of surface of the experimental bead in 180 days

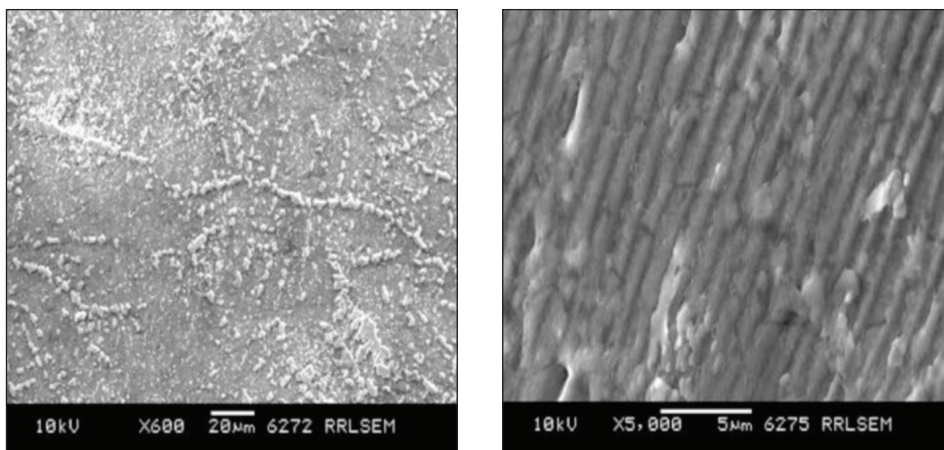


Fig. 4.6 SEM images of surface of the experimental bead in 270 days

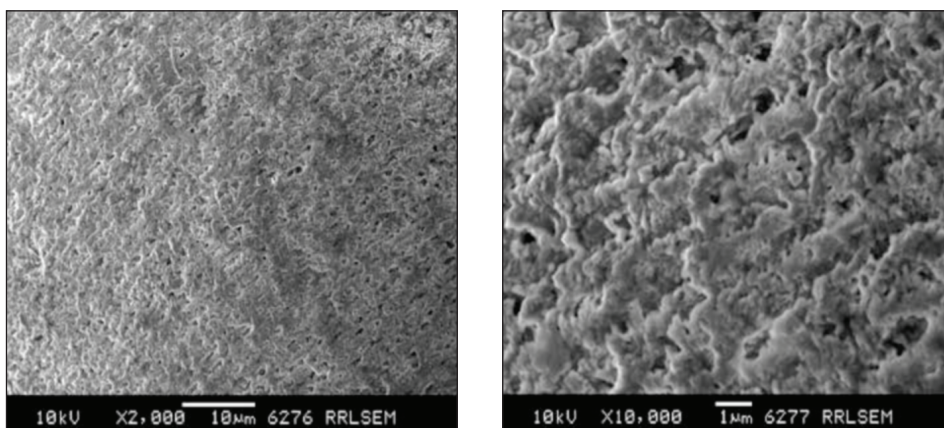


Fig. 4.7 SEM images of surface of the experimental bead in 365 days

In vitro organ culture by using mantle tissue of *P. fucata*

Characterization of nacre layer formation

Scanning Electron Microscopy

Pearl sac with the mantle tissue of *P. fucata* under SEM is given in Fig. 4.8. Shell beads in organ cultures of mantle tissue of pearl oyster *P. fucata* are taken out at different intervals namely 30, 90, 180, 270 and 300 days and the nacre growth is assessed in SEM. Pattern of growth near to the place where explant is attached is shown in Fig. 4.9. A. Irregular pattern of early nacre layer formation is clearly visible in Fig. 4.9. B. Aggregation of crystals in a more orderly manner is visible at higher magnification on another region of bead (Fig.4.9. C). Thick organic layer and greater interface curvature is visible in these early nacre layer formation.

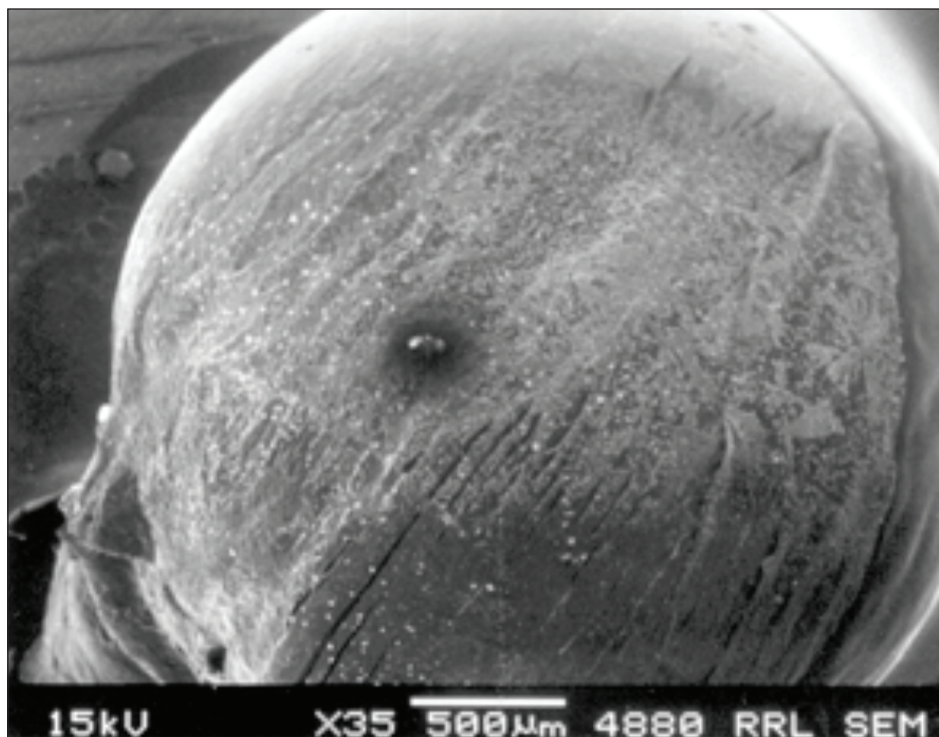


Fig.4.8 SEM image of pearl sac with mantle tissue of *P.fucata*

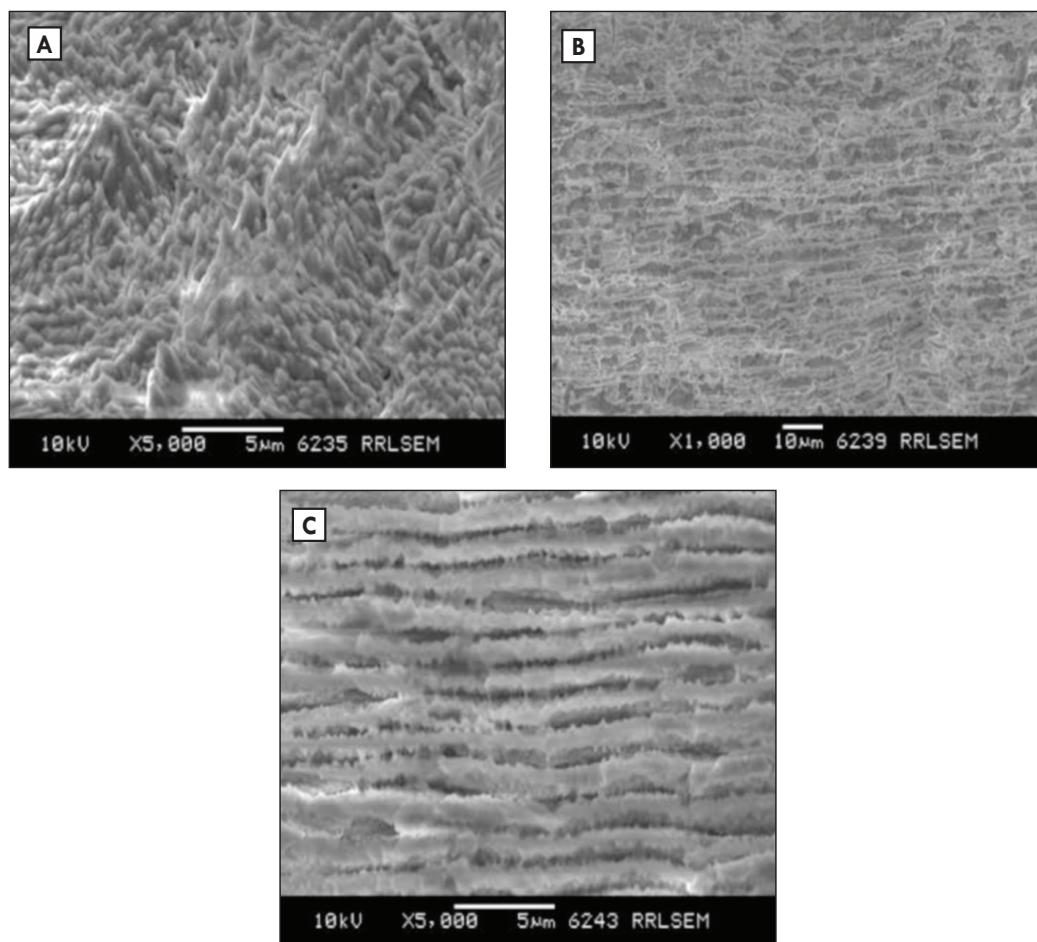


Fig. 4.9 SEM images of surface of the experimental bead in 30 days

Irregular crystal tablets and its growth to form the nacre layer by aggregation of crystallites is visible on one side of the bead in Fig. 4. 10. A. Thick stacking of nacre tablets to form the nacre layer, sandwiched between thin organic matrix and its transition to a 'brick and mortar' like formation is seen in Fig. 4. 10. B and C.

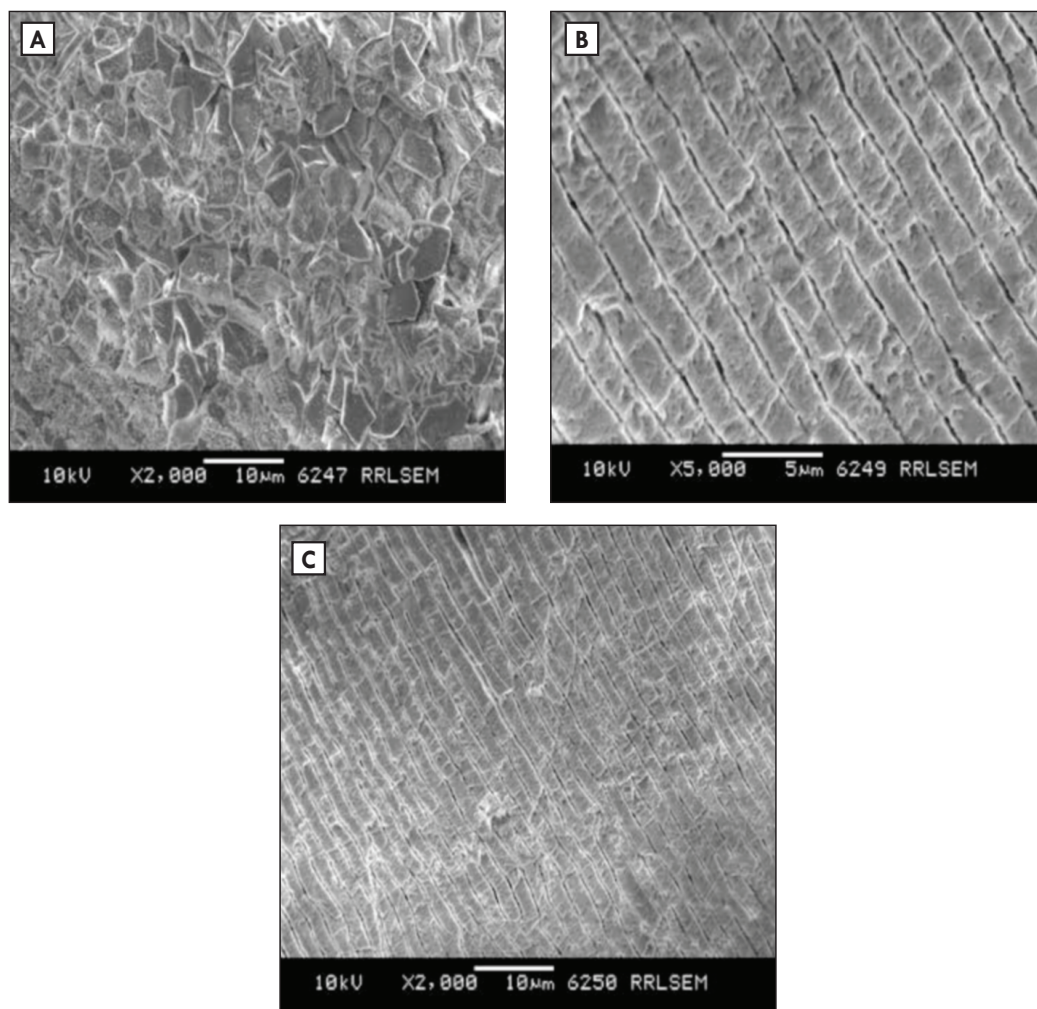


Fig. 4.10. SEM images of surface of the experimental bead in 90 days

More dense space-filling nacre tablet to form a continuous nacre layer is visible in Fig.4.11.A and it is clear at higher magnification in Fig.4.11.B.

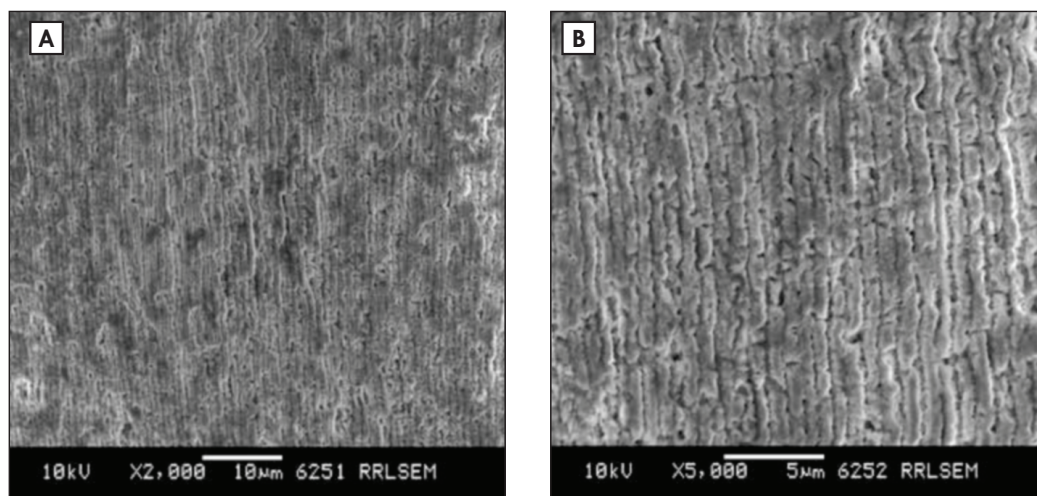
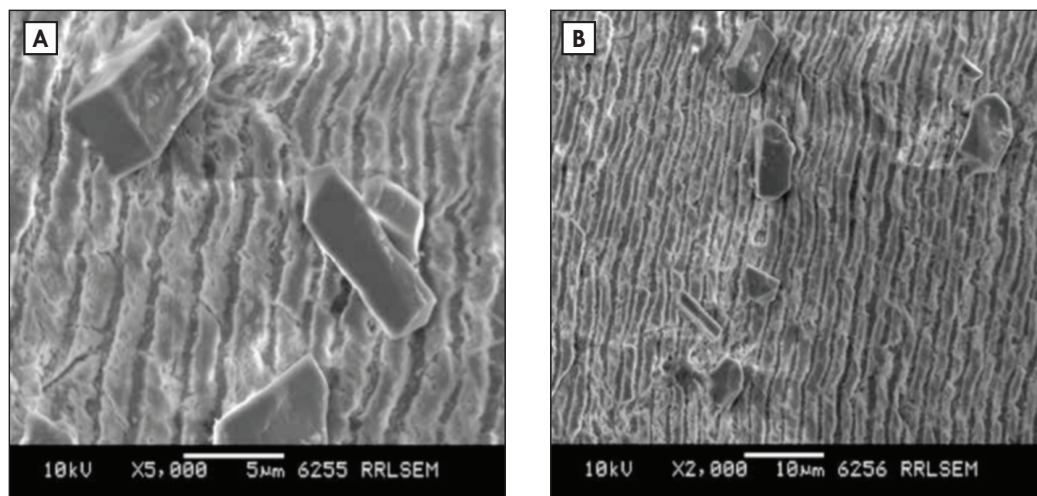


Fig. 4.11 SEM images of surface of the 180 days experimental bead

After 270 days, individual crystal tablets begin to develop as additional layers; matrix layers are wider than the matrix layers in 180 days (Fig. 4.12. A and B). The merging and fusing of nacre layers to form a distinct mature nacre layer is seen on another region of the bead (Fig 4.12. C). The growth of nacre layer in 300 days is shown in Fig. 4.13. A and at a higher magnification in Fig. 4.13. B. Additional tablets secreted over the merged layers during longer period prove the continuous growth of nacre.



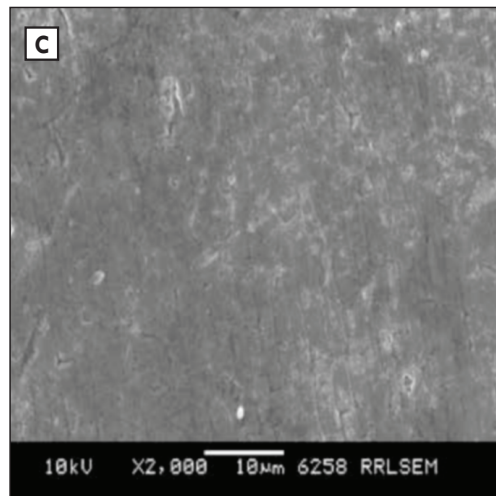


Fig. 4.12 SEM images of surface of the 270 days experimental bead

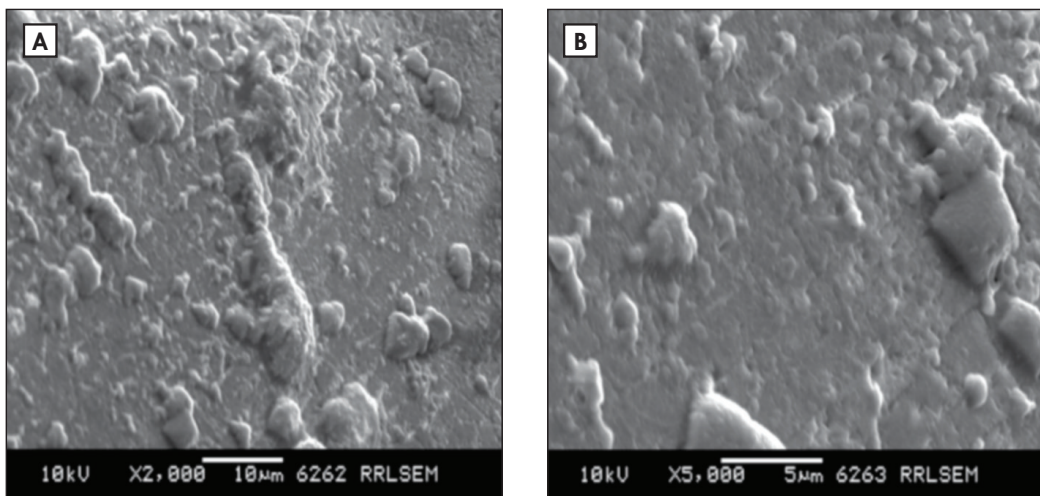


Fig. 4.13 SEM images of surface of the 300 days *in vitro* experimental bead

SEM images of *in vivo* cultured pearl as shown in Fig. 4.14 A and B show a very similar growth pattern of mature nacre as in *in vitro* experimental bead after 300 days.

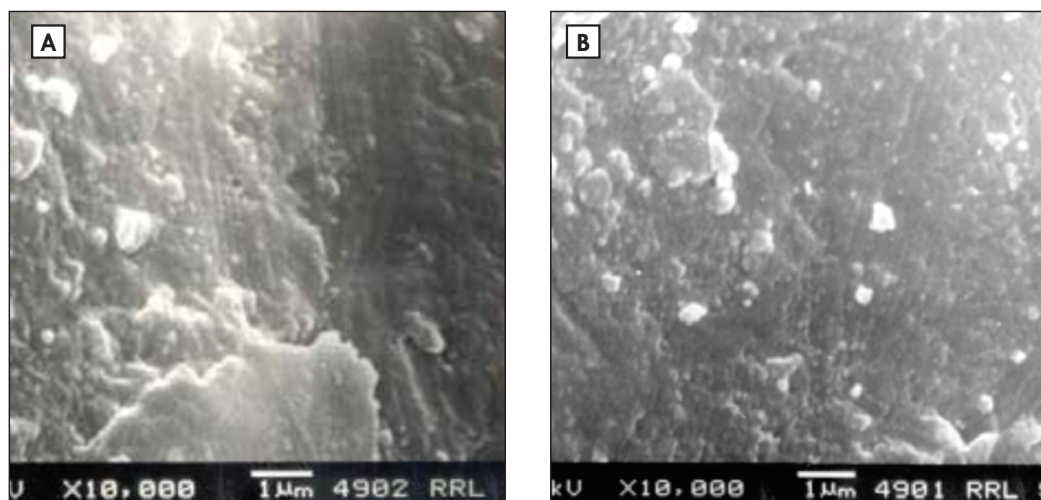


Fig. 4.14 SEM images of surface of the *in vivo* cultured pearl

In vitro* organ culture by using mantle tissue of *P. avicular

Characterization of nacre layer over the bead

Scanning Electron Microscopy

Shell beads in organ cultures of mantle tissue of *P. avicular* are taken out after 3 months. The nacre growth over the bead is analysed using SEM. The results indicate that, there is a continuous growth of nacre coating all over the bead. SEM image of shell bead with nacre deposition is shown in Fig. 4.15.

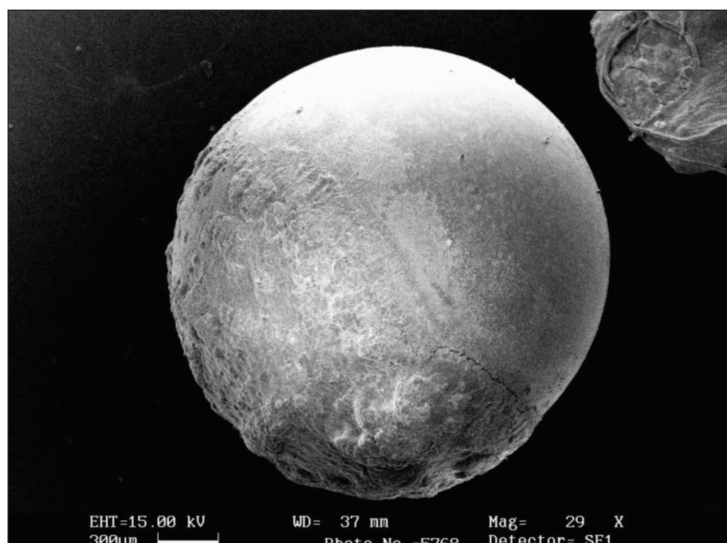


Fig. 4.15 SEM image of experimental bead with *P. avicular* mantle tissue

Crystals develop from the sub microscopic nuclei secreted by the cells over the bead and are embedded in the organic matrix, which is the mechanism of nacre layer formation (Figure 4.16). Characteristic 'brick and mortar' nacreous layer is clearly visible after 90 days. Crystals deposited on the organic matrix increase in size, come into contact with one another and finally develop into a lamellar structure. An organic membrane is sandwiched between them when a crystal joins with adjacent ones and it is found as a groove on the surface (Fig. 4.17).

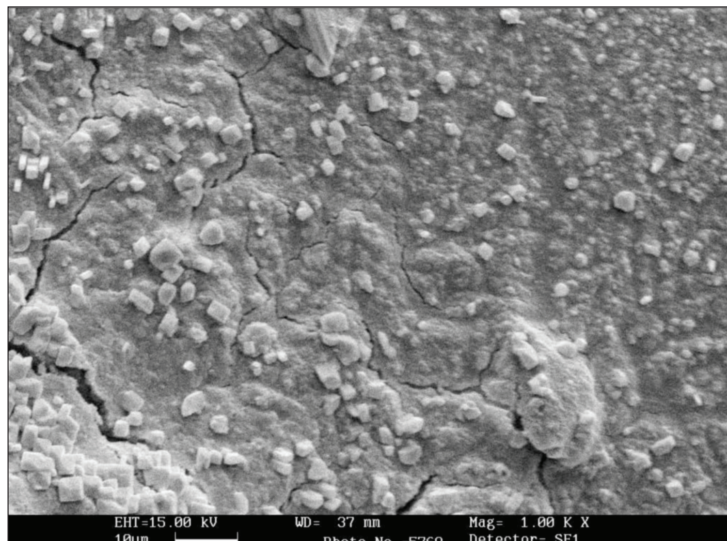


Fig. 4.16 SEM of the crystal nucleation over the experimental bead

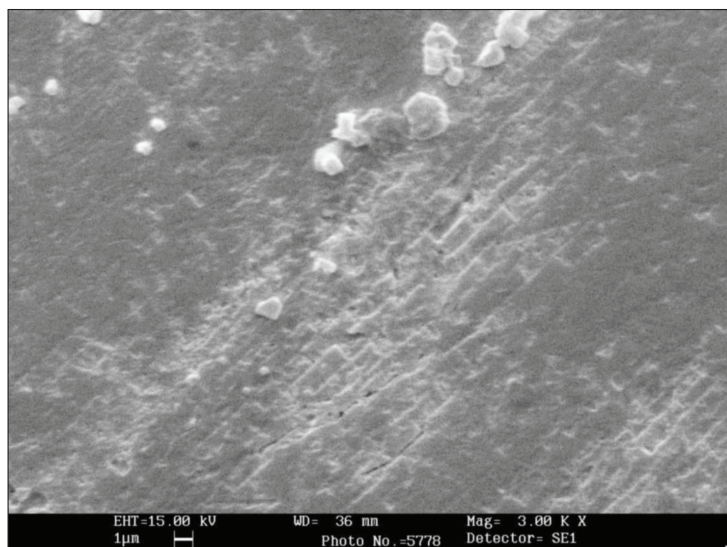


Fig. 4.17 SEM image of 'brick and mortar' structure over the experimental bead

Characterisation of nacre layer by Raman Spectroscopy

Control bead

The Raman spectrum of control bead identifies the biogenic calcium carbonate phase of the skeleton (Fig. 4.18). The control bead shows the aragonite peaks at 207 cm^{-1} , 702-706 cm^{-1} and 1086 cm^{-1} . Moreover, the intensity overtone is very low (0.12).

H. varia

The Raman spectrum of experimental bead identifies the biogenic calcium carbonate phase of the skeleton (Fig. 4.19). The experimental bead shows the aragonite peaks at 206 cm^{-1} , 702-706 cm^{-1} and at 1086 cm^{-1} with the presence of high intensity overtone at 0.14.

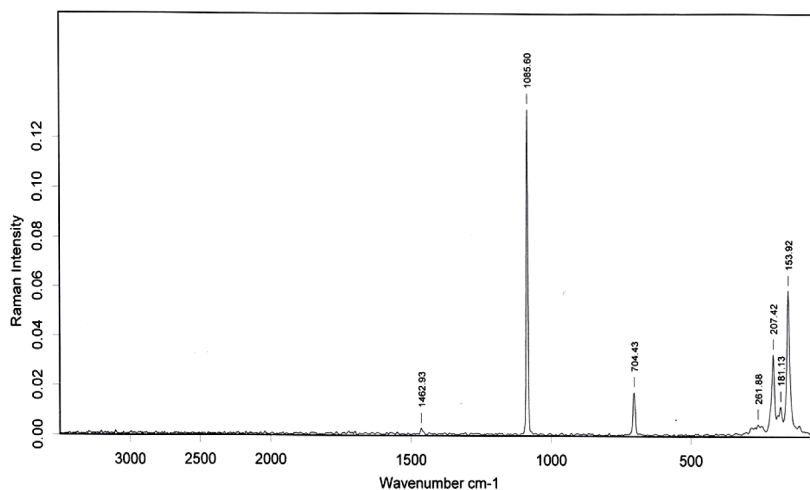


Fig. 4.18 Raman spectrum of control bead

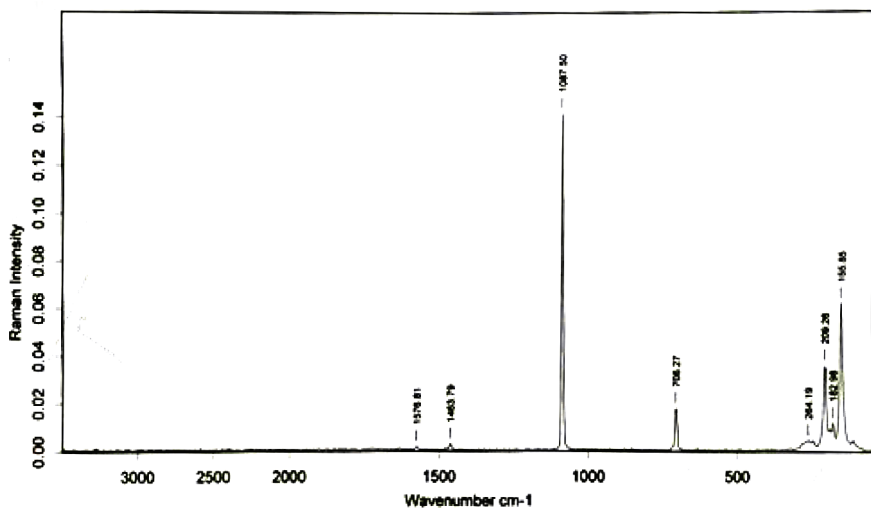


Fig. 4.19 Raman spectrum of experimental bead of *H. varia*

P. fucata

The Raman spectrum of experimental bead identifies the biogenic calcium carbonate phase of the skeleton (Fig. 4.20). The experimental bead shows the aragonite peaks at 206 cm^{-1} , 702-706 cm^{-1} and at 1086 cm^{-1} with the presence of high intensity overtone at 0.25.

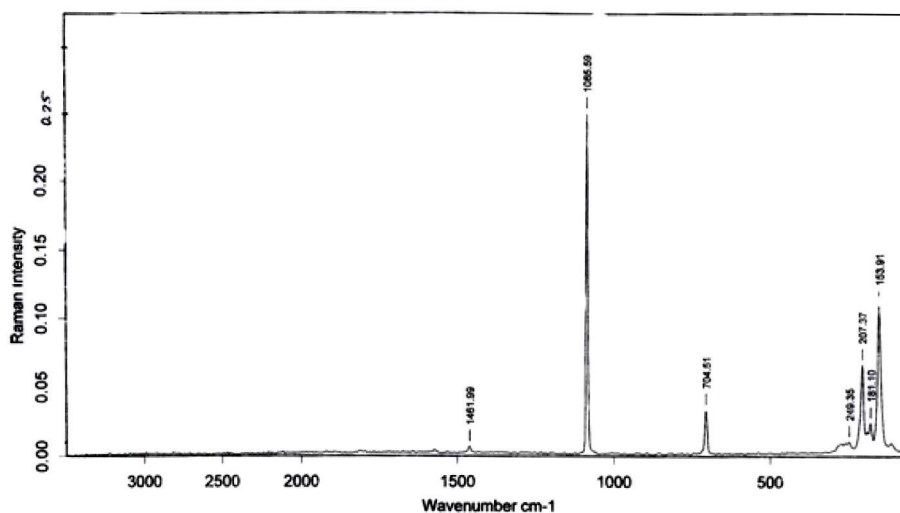


Fig. 4. 20 Raman spectrum of experimental bead of *P. fucata*

P. avicular

The calcium carbonate crystals are further confirmed by Raman spectroscopy based on the Raman shift obtained at 206 cm^{-1} , 702-706 cm^{-1} and at 1086 cm^{-1} with the presence of high Raman intensity overtone at 0.195 in experimental bead (4.21)

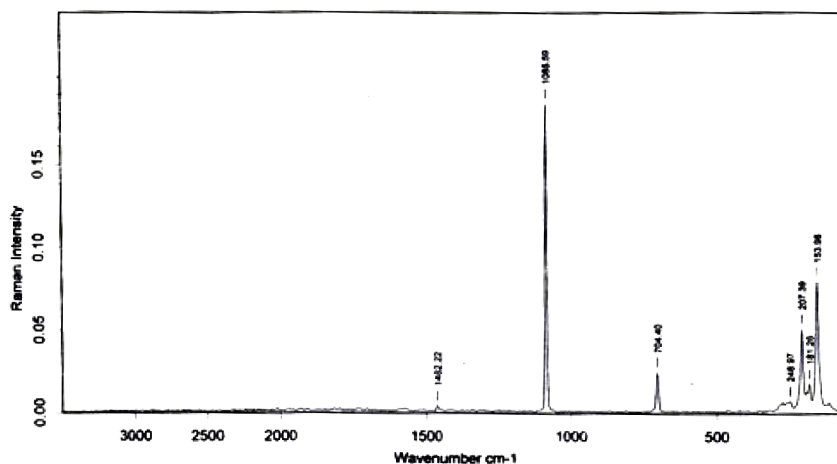


Fig. 4.21 Raman spectrum of experimental bead of *P. avicular*

Characterisation of nacre layer over the bead by Energy-dispersive X-ray spectroscopy

H. varia

The calcium carbonate coating on the experimental bead of *H. varia* is analyzed by EDS (Table 4.1). The bead is rich in calcium (Ca) with the element weight of 51.00% in 300 days of experimental bead.

P. fucata

The calcium carbonate coating present on the *P. fucata* is analyzed by EDS (Table 4.1). The bead is rich in Ca with the element weight of 56.29% in 300 days of experimental bead. Table 4.1 also shows the EDS report of *in vivo* cultured pearl and control beads.

P. avicular

Table 4.1 shows the EDS report of the experimental bead of *P. avicular*. It is revealed that these crystals are rich in Ca with the element weight of 32.11%. These results clearly reveal that the calcium carbonate coating is fashioned over the bead by the biomineralization mechanism of mantle tissue of *P. avicular*.

Table 4.1 Comparative EDS results of control and experimental beads with natural and cultured pearl

Beads	Calcium content (Wt%)
Control Bead	22.75
<i>P. avicular</i>	32.11
<i>H. varia</i>	51.08
<i>P. fucata</i>	56.29
Natural pearl	66.05
Cultured pearl	41.00

Among all the experimental beads, the maximum calcium coating is observed in *P. fucata* followed by *H. varia* and it is more than that on control bead. Moreover, the Ca content in *P. fucata* and *H. varia* is higher than cultured pearl and it reaches the maximum in natural pearl.

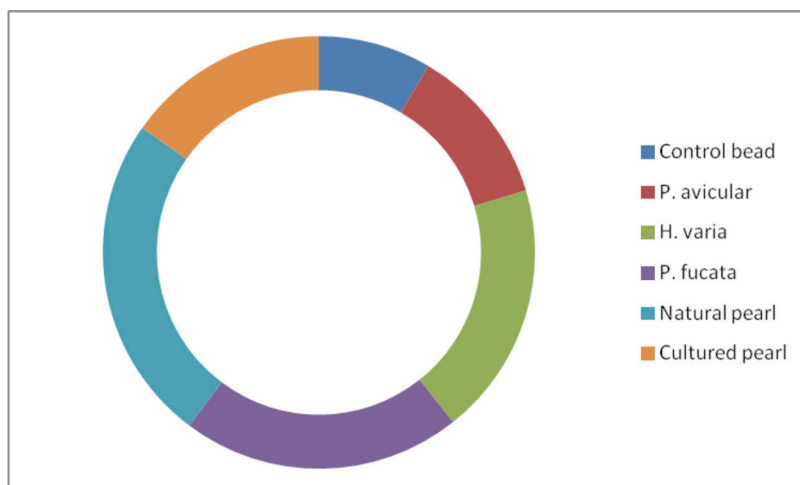


Fig. 4.22 Percentile variation between natural pearl and other *in vivo* and *in vitro* pearls

Since last two decades, a number of publications have reported the establishment of primary cell cultures from marine molluscs and their use for physiological and biochemical aspects of calcification (Machii & Wada, 1989; Lebel *et al.*, 1996; Awaji and Suzuki, 1998; Sudo *et al.*, 1997; Poncet *et al.*, 2000; Suja & Dharmaraj, 2005; Suja *et al.*, 2007, 2014; Gong *et al.*, 2008a, b). Significant progress has been made in the identification and maintenance of cell types involved in skeleton biomineralization and the function of epithelial cells is better understood (Auzoux Bordenave *et al.*, 2007; Suja *et al.*, 2007). Suja and Dharmaraj (2005) achieved coloured crystals by *in vitro* culturing of mantle tissue of the abalone.

In this present work, cell proliferation, its characteristics and crystal formation in mantle explant cultures of different molluscs are studied and compared with the result of *H. varia* reported by Suja and Dharmaraj (2005). Machii, 1974 reported the *in vitro* culture of mantle epithelium of *P. fucata* resulted in sheet-like accumulations of a large number of migrated cells derived from the explant consisting of roundish epithelial cells, pigmented epithelial cells, spindle-shaped or string-like muscle cells and deposition of organic substances. Group of rhombohedral crystals are noticed in *P. fucata* and *P. viridis* as in *H. varia* and amorphous stage of crystals noticed in *P. placenta* which is explained in Chapter 3. Daugavet and Blinova (2015) reported rhombohedral crystals in mantle cultures of *Mytilus edulis* after four months. This work justifies the report of Wada (1985) that the small spherulite of calcium carbonate grew after four days of operation. In this work, microbial contaminants are also identified. Suja *et al.* (2014) studied the effect of antimicrobial agents in cell proliferation of the mantle tissue of *H. varia*. Suja *et al.* (2017) reported the effect of different media in the cell proliferation of *P. viridis*. In this book, cell proliferation and characteristics are studied with different molluscan species with Medium 199 (Chapter 3).

Calcium is detected in all the crystals examined. Morphology and size of the crystals varied in different cultures and in the same culture. Wada (1961) pointed out that the shell substances are crystallized out after passing through the amorphous state when the solid phase separates from the liquid phases. The shell mineralization in Gastropoda and Pelecypoda is assumed to pass through the following three processes: (1) the formation of organic matrix as the basis of shell material (2) fixation of calcium in this organic matrix and (3) the deposition of calcium carbonate crystal. Wilbur (1964) reported that crystals were first seen as round or elongate crystal seeds on the matrix or on the surface of crystals. In the present study of explant cultures, the free crystals by *in-situ* examination, show high birefringence under polarized light and are hexagonal in shape. Wada (1961) reported that the twin of aragonite exhibited regular hexagonal form, which consisted of three grains joining together on its twinning face. It exhibited the twinning types of aragonite at all times during growth of the nacre in pearl oyster shell.

The rhombohedral crystals show high birefringence (chapter 3). Under polarized microscope, aragonite nature is clearly reflected. In the present work, both calcite and aragonite crystals are seen in the same culture flask in some experiments. Aragonite, under ordinary conditions of temperature and pressure, is relatively unstable and changes to calcite, although the rate of change is very slow. Crystals of aragonite have been observed which have completely changed to its stable polymorph calcite (Ford, 1932). Wada (1961) stated that the fixation of calcium and the orientation of mineral crystals were governed directly or indirectly by the elongation of epithelial cells of mantle tissue in molluscan shells and the crystals grown freely from each other exhibited rhombic, irregular or circular shape. In the present study, the cultured cells of mantle of abalone secreted oolite, hexagonal and rhombohedral crystals of calcium carbonate with extreme birefringence showing their calcite and aragonite nature.

Previous studies have shown that primary explant cultures from the mantle were suitable *in vitro* models to study the process of biomineralisation, maintaining cell viability and cell-to-cell interactions (Kleinschuster *et al.* 1996). In this work, pearl sac formation over the bead and crystal formation are studied. Wada (1961) reported that aragonite crystals scattered on the nacreous surface increased in size, came into contact with one another and finally developed into a thin crystal lamella. An organic membrane is sandwiched between them when a crystal joins with adjacent ones, and is found as groove on the inner nacreous surface. It is clearly confirmed by this study.

Calcium carbonate of Haliotidae shells has one layer of calcite and one or more layers of aragonite (Boggild, 1930; Lutts *et al.*, 1960; Wada, 1961; Wilbur, 1964). Machii and

Wada (1989) also reported the strong calcium peaks in *in vitro* crystals in the explant cultures of *P. fucata* with high calcium content optically showing strong birefringence. Samata *et al.* (1994) reported the SEM observation and EDS analysis of crystals over the black secretion and found that these types of crystals were rich in calcium. Calcium is detected in all the examined beads and clearly proved that these are purely calcium carbonate crystals.

Wada (1985) reported that the small spherulite of calcium carbonate grew after four days of operation. Crystal nucleation over the organic matrix and the joining of these crystals results in lamellar formation over the bead (Checa *et al.* 2006). The present study is very similar to the results of the nacreous layer formations reported by Watabe (1981), Blank *et al.* (2003), Zhang and Li (2012). Awaji and Machii (2011) reported the inoculation of mantle cells over the implanted nuclei in oyster and studied the nacre development. Liu *et al.* (2012) studied the nacre layer formation from 5 to 35 days after implantation in oyster. The report of Dharmaraj and Suja (2006) on the *in vitro* nacre formation at different periods using pearl oyster and abalone mantle tissue is the first report in this line.

In this study, calcium is detected more in experimental beads than in control bead by EDS and it clearly indicates that these are purely calcium carbonate crystals. Rhombohedral shaped crystals in the explant cultures and the crystals in the organ cultures are almost similar in its calcium content. Morphology and size of the crystals varied. It is found from the analysis of different experimental beads that all beads are coated with calcium carbonate. The more percentage of calcium on experimental beads than on control bead proves the coating over the beads. It is also noted that, the calcium percentage vary with different experimental beads and also at different positions of same bead. It may be the organic matrix deposition at different periods for the formation of lamellar layer as proposed by Liu *et al.* 2012. The mantle tissue is responsible for shell formation and secretes a matrix complex that includes proteins, polysaccharides and lipids. Crystal nucleation over the organic matrix and the joining of these crystals results in lamellar formation over the bead. The present study is very similar to the results of the nacreous layer of *Halotis* sp. reported by Watabe (1981).

The aragonite crystals are further confirmed by Raman spectroscopy based on the Raman shift in the bead with the mantle tissue of *P. fucata*, *P. avicular* and *H. varia* obtained at 206 cm^{-1} , $702\text{-}706\text{ cm}^{-1}$ and at 1086 cm^{-1} with the presence of high intensity overtones at 0.25, 0.195 and 0.14 which is very similar results to the analysis report of pearl (Bergamonti *et al.* 2011) and the aragonite crystallization studies of Thachepan *et al.* (2006); Suzuki *et al.* (2009); and Dong *et al.* (2010). Raman Spectrum is defined by the position and intensity of the peaks. Peak positions are a function of the force constants and are constant for a given material, even with the variations in incident wavelength. Here it is true that the control bead

made up of calcium carbonate shows the same peaks. Intensities however vary with incident wavelength, direction of polarization of the beam and orientation of the crystal. In control bead, the intensity is observed at 0.12 and in experimental beads 0.14, 0.195 and 0.25 which confirms the biomineralization of CaCO_3 over the bead.

Bersani and Lottici (2010) reviewed the use of Raman spectroscopy for routine investigation in the study of gemstone, its identification and evaluation of composition and genesis of gems. This work justifies these findings and proves the nacre or aragonite coating over the shell beads by its characteristic peaks. The characteristic aragonite peaks are not found on beads of glass, teflon or silica and so it is proved that shell bead is the suitable material for coating in *in vitro* conditions. Scharf and Singer (2003) developed a model for quantifying the thickness of thin coatings and found systematic variations in the Raman peak intensities versus thickness. Sovany *et al.* (2009) proved Raman spectrometry as a suitable method for the estimation of film thickness. The changes in the intensities on experimental shell beads and control bead confirm the thickness of coating. The beads cultured with *P. fucata* mantle tissue show more intensity and confirm more coating.

This study is the first authenticated work of nacre layer formation in *in vitro* conditions with mantle cells of *P. fucata*, *H. varia* and *P. avicular* over bead with newly derived medium (Medium 199). Most of the earlier reports of nacre layer formation were from the shell regeneration studies. Having achieved a success in the basic technology in *in vitro* nacre formation, where the formation of nacreous layer is demonstrated, further experiments are conducted to study whether the nacre growth is continuous throughout the culture period or the growth ceases at a particular period of time in *in vitro* conditions. This was suggested by Awaji and Machii in 2011. In the present work, nacre growth upto 365 days are reported in *H. varia* and upto 300 days in *P. fucata*. However, at present the natural stock of *H. varia* is highly depleted in Gulf of Mannar. Hence the continuation of the work in this species could not be carried out.

Our initial studies revealed the potential of M199 (Suja and Dharamaraj 2005; Suja *et al.* 2007). However, nacre growth in natural media with supplements like WSM and ISO is tested to study its effects on nacre formation. Medium derived by incorporating algal powder in SSW showed significant cell proliferation, cell count and %wt of calcium in nacre coating. Previously, L-15 media was tested for its quality in promoting cell yield and cell adherence by Wen *et al.* (1993a, b). It was found that L-15 medium was a suitable basic medium for *in vitro* cell cultures of marine molluscs. Individual basal medium has limited potential in cell yield. The growth promoting activity of the serum used for medium supplementation in the primary mantle tissue culture was investigated by Cornet (2006) and reported that best

results were obtained using 20% chicken serum and 30% fetal calf serum. In the present study, uniform mineralization is obtained when the bead with mantle tissue is cultured in media with sea water and microalgal powder without serum.

It has been suggested that the insoluble proteins provide the framework and mechanical properties of the shell, whereas the soluble proteins and matrix are involved in crystal nucleation and growth (Belcher *et al.* 1996). In this study, beads treated with WSM and seawater, showed very uniform crystal deposition, but calcium deposition is less in these two combinations due to the initiation of crystal formation over the bead. These crystals will form a uniform lamellar structure of nacre.

The outer calcite prismatic layer is always related to the proteins secreted from the outer epithelial cells of the edge of the mantle, whereas the inner aragonite nacreous layer is related to the proteins of the pallial region (Liang *et al.* 2014). Role of proteins in the biomineralization is not studied in the present work.

Hovden *et al.* (2015) investigated the transition of nacre formation and found that early nacre is distinct with irregular layer thickness, greater layer interface curvature, higher polycrystallinity and frequent stacking faults. As growth continues, more highly packed aggregate regions become continuous nacre plates. In the present study this formation is clearly visible. This also justifies the study of natural pearl from *P. fucata* by Suja *et al.* (2018).

By these studies, it is proved that the mantle cells of pearl producing molluscs are capable of secreting calcium carbonate crystals of high birefringence in *in vitro* conditions. The role played by the microenvironment on the formation of aragonite and calcite crystals needs further investigations. The crystals developed in the *in vitro* cultures are the secretion of the mantle tissue of molluscs. It is essential to continue the study about the interventions of various supplements of nutrient media, stimulation of cells activity by biotic and abiotic stress, molecular methods for genetic improvement of cells, cryopreservation of the cells to make it possible to achieve the lustrous *in vitro* pearl for commercialization.

Summary

This study is the first authenticated work of nacre layer formation in *in vitro* conditions with mantle cells of *P.fucta*, *H.varia* and *P. avicular* over bead. The earlier reports of nacre layer formation were from the shell regeneration studies. Studies with different species of pearl producing molluscs reveal that, all the species are capable of producing cells, pearl sac and nacre crystals by this *in-vitro* technique. Hence it is confirmed that this technology has the potential to pave the way for the production of pearls of various colours from different species of molluscs. Formation of pearl layers or nacre layers is proved by Raman spectroscopy, SEM and EDS analyses in all the experimental shell beads. It is observed that pearl sac can also be formed from the free cells liberated from explant cultures. Southgate *et al.* (2008) in his book 'Pearl oyster' remarked that, it will diminish the pearls 'mystique' when pearl production is successful through *in vitro* methods. The experiments conducted here reveal that shell bead is the best material for nacre adhesion. It is possible to produce pearls through this *in vitro* technique (Fig. 4.22). However, further studies are required to produce more lustrous pearls for commercialization.

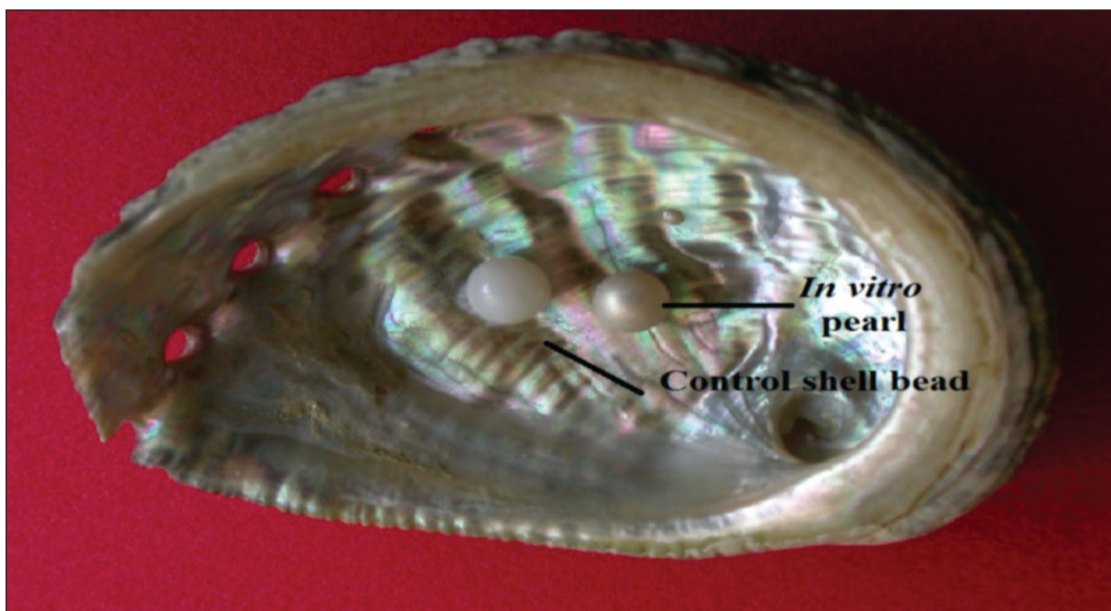


Fig. 4.22 First *in vitro* pearl from *H. varia*

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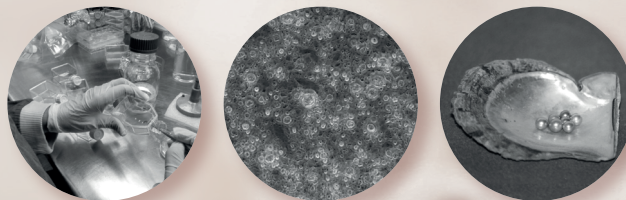
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Antibiotic solution	<i>Haliotis varia</i>	Pseudopodia
Aragonite	Hyalinocytes	<i>Pteria avicular</i>
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Fibroblast	Pearl sac	



In vitro Pearl Culture Techniques

A Biotechnological Approach

This book deals with the set up of tissue culture laboratory, protocols for maintaining a laboratory and methods for different experiments for nacre layer formation to achieve *in vitro* pearl formation. This book also explains the mantle tissue culture techniques of different pearl producing molluscs, cell proliferation with pearl sac formation and crystal deposition which form the basis for *in vitro* pearl formation. The techniques and methodologies used for pearl sac formation are unique and achieved after several trials and errors. Studies with different species of pearl producing molluscs reveal that, all the species are capable of producing cells, pearl sac and nacre crystals by techniques used in this study. Moreover, results of this study revealed that the shell bead is the best material for crystal adhesion. Formation of nacre layers is proved by Raman spectroscopy, Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS) in the experimental shell beads. It is the first authenticated report of nacre layer formation over the shell beads in *in vitro* conditions with mantle cells of different pearl producing molluscs such as *Pinctada fucata*, *Haliotis varia* and *Pteria avicular*. This study is the stepping stone and needs further research to produce more lustrous pearls for commercialization.



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